Anti-inflammatory Effect of Genistein in Human Endometrial Cell Line Treatment with Endotoxin Lipopolysaccharide

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Objective: *The present study aimed to investigate the anti-inflammatory effect of phytoestrogen genistein (Ge) on the secretion of interleukin 6 (IL6) under lipopolysaccharide (LPS) stimulated conditions in human endometrial epithelial cell line RL95-2. The effects of Ge on expression of TLRs 2, 3, 4 and 9 proteins in response to the inflammatory development induced by LPS were also examined and compared with those of 17β-estradiol (E_2).*

Material and Method: *The RL95-2 cells were cultured in the estrogen-deprived media with or without bacterial endotoxin LPS 30 min prior to incubation with Ge (10^{-7}, 10^{-6} or 10^{-5} M) or E_2 (10^{-9} M) for 48 h. The culture media were collected at 1 and 24 h for IL6 measurement by the enzyme linked immunosorbent assay and the cell lysate for TLR protein expression analyzed by semi-quantitative Western blot.*

Results: *Ge or E_2 did not alter the IL6 level in the absence of LPS; however, treatment with either Ge or E_2 for 1 h up to 24 h decreased the IL6 level stimulated by LPS. The cells challenged with LPS significantly upregulated the TLRs 2 and 9 but suppressed the TLRs 3 and 4 protein expression. Forty eight h-treatment with Ge had no effect on the TLRs expression, whereas E_2 down-regulated the increased TLR9 induced by LPS.*

Conclusion: *Ge suppressed the inflammatory response by decreasing the IL6 level, but not associated with the alteration of TLRs-mediated pathway inducedby bacterial infection. In contrast, E_2 decreased the IL6 secretion possibly due to the opposition on LPS-induced TLR9 expression. This provides the potential evidence of soy isoflavone genistein in alleviating the inflammation of endometrium following bacterial invasion.*

Keywords: *Soybean phytoestrogen, Toll-like receptor, Interleukin 6, Endometrium, Estrogen, RL95-2*

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Inflammation induced by either viral/microbial infection or mechanical trauma of the tissues leads to many reproductive organ disorders, including infertility and cancers. The tissue remodeling, i.e. stimulating cell proliferation and differentiation induced by cytokine released during the inflammatory process are suggested for the cause of disorders[1,2].

The inflammatory cytokines Interleukin 6 (IL6) is a pleiotropic cytokine which plays a crucial role in immune physiology and pathology of reproductive functions. However, it is in homeostasis and controlled by hormonal feedback mechanisms[1]. Loss of the negative feedback by ovarian sex steroid estrogen, resulting in the elevation of IL6 level even in the absence of microbial components, has been found in postmenopausal women. The excessive IL6 production has been suggested to be a major cause of pathogenesis and phenotypical changes of endometrium in several diseases such as endometriosis and endometrial cancer[2]. Pharmacological modulation of IL6 synthesis and release using estrogen or phytoestrogen supplements may be a good strategy in restoring immune homeostasis and preventing this disorders.

In general, a key regulator in IL6 gene expression is the transcription factor NF-κB, of which its activity is regulated through the signaling of the pattern recognition receptors (PRRs) pathway. Toll-like receptors (TLRs) are the prominent PRRs found on a membrane of specific immune cells and epithelia. They mediate the cytokine secretion by recognition and binding to pathogen-associated molecular patterns

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(PAMPs) molecules of microorganisms. At least TLRs 1-10 have been characterized in human. In human endometrial tissues, the gene expression of TLRs 2, 3, 4 and 9 have been demonstrated with the variation through the menstrual cycle and suggested to correlate with the cyclic changes of estrogen levels[3]. In theory, TLRs 2, 4 and 5 recognize bacterial components, such as lipopolysaccharides (LPS) and/or flagellin, whereas TLR3 recognizes double strand nucleotide sequences of microorganism[4]. The ascending contamination with microbial component LPS of pathogenic *E. coli* and *E. fecalis* colonized in the area near the urethral orifice has frequently occurred in women. Postpartum infection with *E. coli* or treatment with LPS has been shown to induce IL6 expression in cow and in primary bovine endometrial cell culture, respectively[5,6]. The mechanism was thought to be mediated by TLR4 and its signaling pathway[5]. However, there is a little study of the IL6 release in response to LPS in human endometrium.

Genistein the isoflavone phytoestrogen is one of the active ingredients and mostly present in soy diet. Its chemical structure is similar to 17β-estradiol, an endogenous estrogen[7]. The anti-inflammatory effect of genistein has also been previously described. In an in vitro study of microglia, genistein inhibits the increased pro-inflammatory cytokines IL-1β and TNF-α induced by LPS[8]. In addition, genistein at concentration of 5-50 μM significantly inhibits the pro-inflammatory cytokines, including IL6 released by the endometriosis cell culture[9]. However, there are controversy between inhibition and promotion of tumorgenesis or endometriosis following phytoestrogen treatment. The dose-dependent effects of genistein, low doses ($\leq 10^{-6}$ M) vs. high doses ($>10^{-6}$ M), related to advantage or disadvantage have been discussed[10]. The restoration of immunological homeostasis by genistein during the inflammatory process in response to microorganisms is also intrigued.

For the reason that the endometrial tissues are commonly contaminated with Gram negative bacteria, the anti-inflammatory effects of genistein on the secretion of inflammatory IL6 and the expression of TLRs 2, 3, 4 and 9 proteins in response to bacterial endotoxin LPS were aimed to investigate.

Material and Method

Material

Genistein, 17β-estradiol, lipopolysaccharide (*Escherichia coli* 0111: B4, purified by phenol extraction), insulin, non-essential amino acid, *L*-glutamine and high purity grade salts were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (DPBS), phenol red-free DMEM, fetal bovine serum (FBS), 0.05% trypsin-0.53 mM methylenediaminetetraacetic acid (EDTA), penicillin-streptomycin and fungizone were purchased from GIBCO BRL (Grand Island, NY, USA). Charcoal-stripped FBS (csFBS) was purchased from Biowest Co. (Miami, FL, USA).

Cell culture

Human endometrial cell line RL95-2 cells (ATCC® Number: CRL-1671™, VA, USA) are epithelial origin with well-defined structures and present both cytoplasmic and nuclear estrogen receptors. The cells (passage number 138-145) were maintained in DMEM containing 5% FBS, 1% non-essential amino acid, 5 μg/ml insulin, 200 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (standard media) and incubated at 37°C in a humidified 5% CO_2 in air. The culture media were changed every two days.

Cell treatment

The RL95-2 cells (10^6 cells) were seeded in a 50-mm cell culture dish. After reaching confluence, they were pre-conditioned in phenol red-free DMEM with 2% csFBS and other supplements similar to the standard media (estrogen-deprived media) for 48 h. The cells were then inoculated with 1 μg/ml of O111: B4 *Escherichia coli* Lipopolysaccharides for 30 min prior to the addition of genistein (10^{-7}, 10^{-6}, 10^{-5} M), 17β-estradiol (10^{-9} M) or DMSO as a vehicle control for 48 h. The 500 μl of each culture media was collected for IL6 level at 1 and 24 h, and at the end of the experiment, the cells were collected for total protein isolation.

Measurement of interleukin 6 secretion

The RL95-2 cell culture media were analyzed for IL6 concentration using Duoset ELISA (R&D Systems, Minneapolis, MN, USA). Briefly, a 96-well plate was coated with capture antibody of IL6 and incubated overnight at 4°C. After non-specific blocking, the plate was incubated with IL6 standard or samples at room temperature for 2 h. Following washing steps, the IL6 antibody was applied for 2 h, washed, and streptavidin-horseradish peroxidase was added and incubated in the dark place for 30 min. Following washing steps, TMB step-1 substrate was added and followed by H_2SO_4 solution to stop reaction. The

concentration of IL6 was calculated from the standard curve by reading an optical density at 450 nm with an ELISA plate reader.

Western blot analysis

Total protein from the RL95-2 cells was extracted with lysis buffer, and determined for concentrations using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., USA). Then 60 µg of total protein per sample was separated on 10% SDS-PAGE (Bio-rad laboratories, Inc) and subsequently transferred to a polyvinylidenedifluoride membrane (Pall Life Sciences, USA). After non-specific blocking, the membranes were incubated overnight with a 1: 200 dilution of rabbit anti-TLR2 antibody, goat anti-TLR3 antibody, rabbit anti-TLR4 antibody, goat anti-TLR9 antibody and housekeeping mouse anti β-actin (Santa Cruz Biotechnology Inc., USA). After washing, the membranes were incubated with anti-rabbit secondary antibody for TLR2 and 4, anti-goat secondary antibody for TLR3 and 9, and anti-mouse secondary antibody for β-actin, which is conjugated with horseradish peroxidase (Sigma Chemical Co). Finally, the immunoreactive blots were detected by clarity western ECL chemiluminescent substrate (Bio-rad laboratories, Inc) according to the manufacturer's instructions. The fluorescent band products were exposed for 8 minutes on Amersham™ hyperfilm ECL (Amersham-Pharmacia Biosciences) in a dark room. The bands density was quantified by Scion Image software (Scion Corporation, Frederick, MD). All experiments were repeated at least three times.

Data analyses

All values are presented as mean ± standard error of mean (SEM), and n is the number of monolayers used in each experiment. The differences between control and experimental means were analyzed using a Student's t-test. The difference among means of more than two groups was analyzed by ANOVA and post hoc test by Student Newman Keuls (Prism 5.0 Graph Pad software, Inc San Diego, CA, USA). A value of $p<0.05$ was considered statistically significant.

Results

Effect of genistein on secretion of IL6 in human endometrial epithelial cells under control and LPS stimulation

Under control condition where the RL95-2 cells were treated with vehicle DMSO, the level of IL6 was undetectable at the beginning of experiment (time 0). After that the IL6 level was detected at 151.13±16.60 pg/ml for the first h and maintained at 136.13±23.31 pg/ml upto 24 h without significant difference between two time points (Fig. 1). Treatment with various concentrations of genistein (10^{-7}, 10^{-6} and 10^{-5} M) or 17β-estradiol (10^{-9} M) did not alter the level of IL6 at 1 and 24 h when compared to the control.

In LPS-inoculated cells, the level of IL6 was significantly increased by 2 folds (343.75±86.64 pg/ml) within 1 h as compared to the non-inoculated cells. The increased IL6 level then declined within 24 h to a somewhat higher level that was not significant different from that of non-inoculated cells at the same time point. However, this level was significantly different from the level at 1 h of the LPS-inoculated cells (221.88±7.20 pg/ml; $p<0.05$ by unpaired t-test) (Fig. 2). A significant decrease in the IL6 level was detected in the LPS-inoculated cells after treatment with some concentrations of genistein or 17β-estradiol for 1 and 24 h when compared to the control LPS-inoculated cells (Fig. 2).

Effects of genistein and 17β-estradiol on the expression of TLR proteins under LPS-stimulated conditions

Our previous study has shown the differential expression of TLRs 2, 3, 4 and 9 proteins in the RL95-2 cells. Treatment with genistein (10^{-7} M) for 48 h was also found to up-regulate the TLR2 protein[11]. We further investigated whether the genistein-decreased IL6 secretion in response to LPS was due to alteration of these TLR proteins in the RL95-2 cells. As shown in

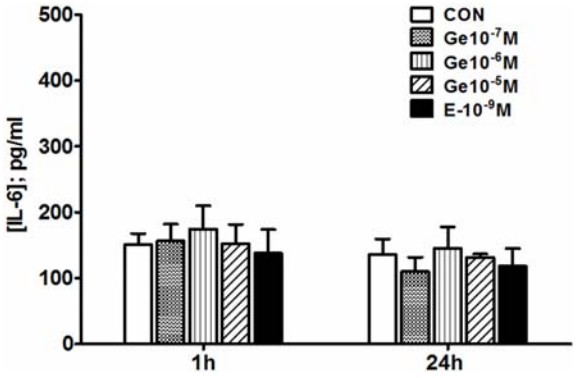

Fig. 1 Effect of genistein and 17β-estradiol on the interleukin 6 (IL6) level in RL95-2 cells. Cell culture media were collected at 1 and 24 h after treatment with genistein (Ge), 17β-estradiol (E) or DMSO (CON) and the IL6 level measured by ELISA. Data are means ± SEM (n = 6).

Fig. 3, the cells treated with LPS alone significantly increased the TLRs 2 and 9 and decreased the TLRs 3 and 4 expression. Treatment with all concentrations of genistein had no effect on the TLRs being studied as compared to the control LPS-treated cells (Fig. 4). However, 17β-estradiol was found to slightly decrease the LPS-induced increase in TLR9 (Fig. 4D).

Discussion

In human, ascending infection from Gram negative bacteria (i.e. *E. coli*) is a major cause of bacterial infection of the female reproductive tract leading to pathogenesis of reproductive tract diseases and infertility. The inflammatory reactions resulted from stimulating the TLRs and releasing the acute phase pro-inflammatory cytokines in response to invading pathogens are of critical importance to the pathogenesis. The IL6 has been indicated to involve because of its pleiotropic action[1,2]. The present study revealed that the RL95-2 cells culturing in the estrogen-deprived media for 1 h upto 24 h were capable of secretion of IL6 under basal condition. This IL6 secretion was not affected by all concentrations of genistein or 17β-estradiol, suggesting its secretion in human endometrial cells is estrogen independent. While 17β-estradiol has been reported to induce TLR activation and promote the production of inflammatory cytokines and immune responses[12], the other study revealed the IL6 production not being regulated by ovarian steroid[13]. Thus, lack of genistein on IL6 secretion under the control condition supports the safety use of this drug.

In contrast to the control condition, an increase in the IL6 level after LPS inoculation for at least 24 h was evident in the RL95-2 cells consistent with that has been reported in endometriosis tissue implants[9] and bovine endometrium[5]. Basically, LPS is an outer membrane of Gram negative bacteria commonly used to substitute bacterial infection. It is recognized by cell surface TLR4 with cooperation with MD2 and CD14 to mediate signal transduction[4]. The finding of LPS-induced IL6 secretion in the human endometrial cell line RL95-2 reflects the capable of glandular endometrial cell itself as an innate immune

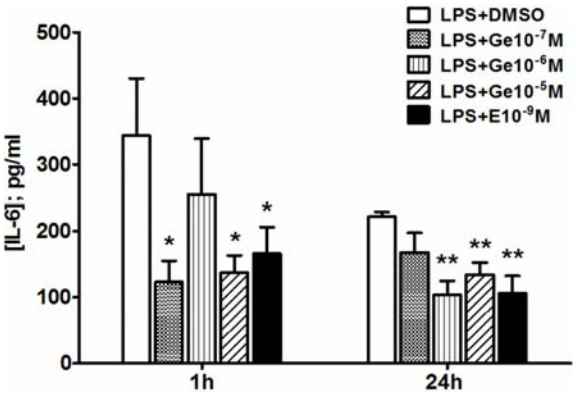

Fig. 2 Effects of genistein and 17β-estradiol on the interleukin 6 (IL6) level in RL95-2 cells inoculated with LPS. The cells were inoculated with LPS (1 μg/ml) for 30 min prior to treatment with genistein (Ge), 17β-estradiol (E) or DMSO (CON). Culture media were collected at 1 and 24 h after treatment with Ge, E or DMSO and the IL6 level measured by ELISA. Values are means ± SEM (n = 6). * or ** respectively denotes $p<0.05$ or $p<0.01$ significantly different from corresponding LPS group using a Student t-test.

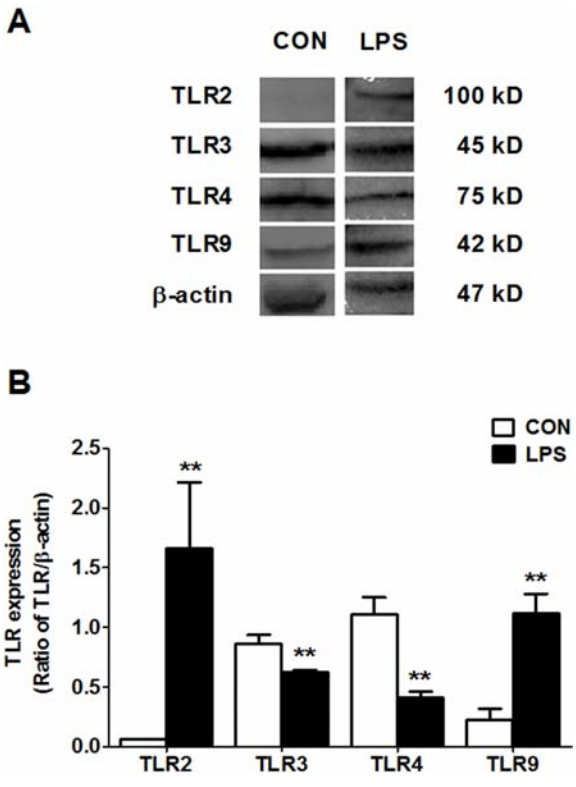

Fig. 3 A) Western blot bands of TLRs 2, 3, 4, 9 and internal control β-actin proteins in RL95-2 cells without or with LPS stimulation. Total proteins were isolated from the cells grown in the estrogen-deprived media in the presence of LPS or vehicle control DMSO for 48 h. B) Densitometric analysis of TLRs/β-actin band. Values are mean ± SEM (n = 3). ** denotes $p<0.01$ significantly different from corresponding control group using a Student t-test.

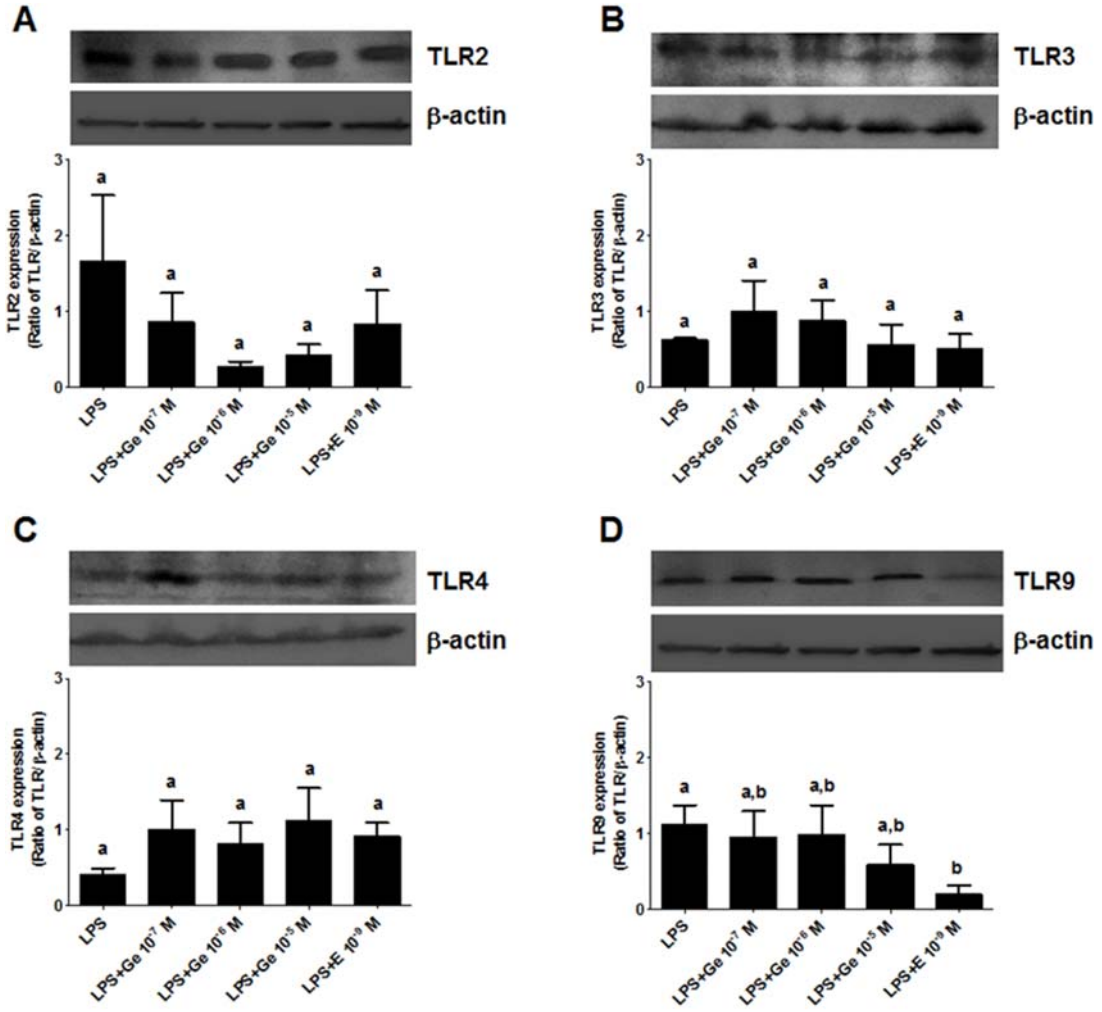

Fig. 4 Western blot and densitometric analysis of (A) TLR2, (B) TLR3, (C) TLR4 and (D) TLR9 to β-actin proteins in response to genistein or 17β-estradiol in RL95-2 cells inoculated with LPS. Total proteins were isolated from the cells grown in the estrogen-deprived media and pretreated with DMSO (Control) or LPS for 30 min followed by genistein (Ge), 17β-estradiol (E) at indicated concentrations for 48 h. Values are mean ± SEM (n = 3). Bars with different letters (a, b) indicated significant differences among groups at $p<0.05$ using ANOVA followed by Student Newman Keuls' post-hoc test.

system.

Treatment with most of the concentrations of genistein being examined or 17β-estradiol for 1-24 h significantly reduced the IL6 level induced by LPS. This finding is in accordance with a previous study of Dijsselbloem et al reporting the effect of genistein on decreasing IL6 secretion in monocyte-derived dendritic cells induced by LPS[14]. The underlying mechanism of genistein has been indicated to suppress TNF-α-induced NF-κB-dependent IL6 gene expression[14]. In addition, due to its phenolic structure, genistein may act as an inhibitor of many enzymes, tyrosine protein kinase in particular, to affect a host's other intracellular processes[14]. The other possible mechanism of genistein may be mediated through the estrogen receptors, since in the current study 17β-estradiol revealed the similar effects as genistein. The anti-inflammatory effects of estrogen as evidenced by decreasing the IL6 secretion induced by LPS has been reported in murine[15]. However, the promoting effects on the LPS-induced IL6 secretion during the menstrual cycle or estrogen and testosterone administration has been shown[16]. Perhaps, the use of genistein rather than estrogen may be chosen to protect endometrial cells and tissues from cytokine-mediated inflammation and diseases following bacterial infection by

suppressing the IL6 secretion.

Even though the LPS-induced IL6 release mediated by TLR4 leads to inflammation, the question as to whether the continuous exposure to LPS enhances or suppresses the innate immune system of endometrium is addressed. Modification or alteration of TLR expression level has been linked to inflammation in many diseases. Chotimanukul and Sirivaidyapong indicated that TLR2 or TLR4 protein expression in glandular endometrium of canine pyometra was higher than those of healthy dogs[17,18]. The high level of TLR2 expression was also found in the healthy dogs at the estrus and diestrous cycle[18]. This suggests that the expression of TLR is under the regulation of ovarian steroid hormones. It is possible that the changes of TLR expression in response to microorganism or hormones may be relevant to many complexities including infertility and cancers.

In the present study, the TLRs 2, 3, 4 and 9 proteins were differentially expressed in the RL95-2 cells. Exposure to LPS was found to significantly increase the TLRs 2 and 9, but decrease the TLRs 3 and 4 expression. Previous study in canine endometrium has been demonstrated that LPS up-regulates TLRs 2 and 4 genes during the diestrous cycle[19]. In correlation with the present study, LPS has been reported to increase TLR9 gene expression through NF-κB, ERK and p38 MAPK signal pathways in macrophage[20] and dendritic cells[21]. The present findings of LPS-induced up-regulation of the functional TLRs 2 and 9 proteins may help promote the immune response to bacterial infection. However, the down-regulation of TLRs 3 and 4 induced by LPS seems to contradict with the recent studies showing that LPS increases expression of TLR4 gene and protein in human endometrial stromal cells[22] and up-regulates of TLR3 in human peripheral blood monocyte and monocytic cell line through a NF-κB dependent TLR4 signaling pathway[23]. However, lack of TLR4 mRNA expression and response to lipopolysaccharide (LPS) has been previously found in the RL95-2 cells[24]. This different result is complicated and possibly due to different cell type, conditioned media and signaling mechanism.

Although the TLRs 2, 3, 4 and 9 proteins were modulated by bacterial endotoxin, these TLR expressions were not affected following treatment with genistein. On the other hand, treatment with 17β-estradiol for 48 h was able to inhibit the LPS-induced increase in TLR9, suggesting a possible role of estrogen in protecting epithelial cells from triggering the production of inflammatory cytokines and chemokines. In the present study, as treatment with 17β-estradiol was found to decrease both IL6 secretion and TLR9 protein expression induced by LPS, it indicates that 17β-estradiol decreases the inflammatory response in human endometrial cells possibly as a consequence of down-regulation of the TLR9.

In conclusion, the present study in human endometrial cells demonstrates the functional expression of TLRs 2, 3, 4 and 9 proteins which are differentially regulated upon stimulation with endotoxin LPS. Although no alterations in the LPS-induced TLRs protein expression, genistein could attenuate the inflammatory responses induced by the bacterial component as evidenced by the decreased level of IL6. By contrast, 17β-estradiol decreased the inflammatory IL6 possibly due to the opposition on LPS-induced TLR9 expression. These data provides the potential evidence of soyisoflavonegenisteinin alleviating the inflammation of endometrium following bacterial invasion.

What is already known on this topic?

TLRs 2, 3, 4 and 9 mRNA and proteins are differentially expressed in human endometrial RL-95 cells. Genistein has been evidenced to up-regulate the TLR2 and modulate the TLRs proteins under viral infection mimicking condition. However, the TLR function through the release of inflammatory IL6 in response to genistein under basal and bacterial endotoxin LPS-inoculated condition remains to be investigated.

What this study adds?

Genistein by itself did not affect the basal level of IL6 but could suppress the inflammatory responses induced by bacterial component as evidenced by decreased major inflammatory cytokine IL6. The anti-inflammatory effect of genistein appeared not to be associated with the LPS-induced alterations in TLRs 2, 3, 4 and 9 protein expression of human endometrial RL95-2 cells. The present findings suggest a potential role of genistein phytoestrogen in the attenuation of endometrial inflammation from Gram negative bacterial infection.

Acknowledgements

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Potential conflicts of interest

None.

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ฤทธิ์ต้านอักเสบของสารเจนิสทีอีนในเซลล์ไลน์เยื่อบุผิวมดลูกของมนุษย์ที่ได้รับสารพิษไลโปโพลีแซคคาไรด์

นรธีร์ บัวทอง, สุทธาสินี ปุญญโชติ, ฉัตรศรี เดชะปัญญา

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์ต้านอักเสบของสารเจนิสทีอีน (genistein, Ge) ต่อการหลั่งสารอินเตอร์ลิวคิน 6 (IL6) ภายใต้สภาวะที่กระตุ้นด้วยไลโปโพลีแซคคาไรด์ (lipopolysaccharide, LPS) ในเซลล์ไลน์เยื่อบุผิวมดลูกของมนุษย์ชนิด RL95-2 รวมถึงผลของ Ge ต่อการแสดงออกของโปรตีน TLRs 2, 3, 4 and 9 ในการตอบสนองต่อภาวะอักเสบจาก LPS โดยเปรียบเทียบผลดังกล่าวกับ 17β-estradiol (E_2)*

วัสดุและวิธีการ: *นำเซลล์ RL95-2 ไปเพาะเลี้ยงในน้ำเลี้ยงเซลล์ที่พร่องเอสโตรเจนที่มีหรือปราศจาก LPS เป็นเวลา 30 นาที ก่อนใส่ Ge (10^{-7}, 10^{-6} or 10^{-5} M) หรือ E_2 (10^{-9} M) เป็นเวลา 48 ชั่วโมง เก็บน้ำเลี้ยงเซลล์ที่เวลา 1 และ 24 ชั่วโมงเพื่อตรวจวัดระดับ IL6 ด้วยเทคนิค enzyme linked immunosorbent assay และเก็บเซลล์ที่ย่อยแล้วเพื่อวิเคราะห์การแสดงออกของโปรตีน TLRs ด้วยเทคนิค semi-quantitative western blot*

ผลการศึกษา: *สาร Ge หรือ E_2 ไม่มีผลเปลี่ยนแปลงระดับ IL6 ในสภาวะที่ปราศจาก LPS แต่การให้สารดังกล่าวเป็นเวลา 1 ถึง 24 ชั่วโมงมีผลลดระดับ IL6 ที่ถูกกระตุ้นด้วย LPS ยังพบว่าเซลล์ที่ถูกกระตุ้นด้วย LPS เพิ่มการแสดงออกของ TLR2 และ 9 แต่กลับลดการแสดงออกของ TLR3 และ 4 อย่างมีนัยสำคัญทางสถิติ การให้ genistein เป็นเวลา 48 ชั่วโมงไม่มีผลปรับเปลี่ยนการแสดง TLR ขณะที่ E_2 ลดการแสดงออกของ TLR9 ในภาวะที่เซลล์ถูกเหนี่ยวนำด้วย LPS*

สรุป: *Ge ยับยั้งการตอบสนองต่อการอักเสบด้วยการลดระดับ IL6 โดยไม่มีผลเปลี่ยนแปลง TLR pathway ที่ถูกกระตุ้นเมื่อมีการติดเชื้อแบคทีเรีย ในทางตรงข้ามผลของ E_2 ที่ลดการหลั่ง IL6 น่าจะเกิดจากการยับยั้งการแสดงออกของโปรตีน TLR9 ที่ถูกเหนี่ยวนำโดย LPS ผลการทดลองแสดงให้เห็นถึงหลักฐานสำคัญของสารเจนิสทีอีนที่จะช่วยลดการอักเสบของเยื่อบุมดลูกจากการรุกรานของเชื้อแบคทีเรียได้*