Soy isoflavones improves endometrial barrier through tight junction gene expression

Pongpat Kiatprasert, Chatsri Deachapunya¹, Chutamas Benjanirat and Sutthasinee Poonyachoti

Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Henri-Dunant Road, Patumwan, Bangkok 10330, Thailand and ¹Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Sukhumvit Road, Bangkok 10110, Thailand

Correspondence should be addressed to S Poonyachoti; Email: sutthasinee.p@chula.ac.th

Abstract

Contamination with bacterial endotoxin causes the disruption of the tight junction (TJ) barrier. We investigated the ameliorative effect of dietary flavonoids genistein (Ge) and daidzein (Di) in normal or lipopolysaccharide (LPS)-induced disruption of epithelial barrier function of the endometrium. Using the immortalized porcine glandular endometrial epithelial cells (PEG), transepithelial electrical resistance (TER) and FITC-dextran flux (FD-4) across the monolayer were measured. The mRNA expression of TJ proteins, zona occludens-1 (ZO1), and claudin-1, -3, -4, -7 and -8 was evaluated by real-time RT-PCR for coinciding effect of Ge or Di occurred at the gene transcription level. The results revealed that Ge and Di altered the TER, depending on times and concentrations. Low concentration (10^{-10} M) of both compounds decreased the TER, whereas higher concentrations (10^{-8} and 10^{-6} M) increased the TER which was not related to the FD-4 flux. The increased TER by Ge or Di was parallel to the induction of *claudin-3* and *-4* or *-8* mRNA expression respectively. With LPS inoculation, all isoflavone treatments inhibited the decreased TER induced by LPS, but only Ge (10^{-8} or 10^{-6} M) or Di (10^{-10} or 10^{-6} M) was coincidence with the decreased FD-4 flux. Under this LPS-stimulated condition, some or all examined TJ gene expressions appeared to be promoted by specific concentration of Ge or Di respectively. Our findings suggest that the soy isoflavones treatment could promote and restore the impaired endometrial barrier function caused by LPS contamination.

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Introduction

Tight junction (TJ) of endometrial epithelial cells employs physical barriers in the regulation of ion transport via a paracellular pathway. The paracellular barrier function provides the tightness against uncontrolled leak flux as well as the maintenance of mucosal monolayer integrity that contributes to endometrial health (Wira *et al.* 2010). The disruption of TJ of endometrial epithelial cells may result in the major implication in reproductive failure, such as infertility, infection, and chronic abortion. TJ is a complex of membrane-bound proteins, occludin and claudins, and their adaptors and scaffolding proteins (e.g., junctional adhesion molecule, zona occludens (ZO)-1, ZO2, and ZO3) (Förster 2008, Van Itallie & Anderson 2013). The largest family of TJ proteins is the claudins. At least 24 members of claudins have been identified. Claudins are 20–27 kDa, tetraspan proteins with relatively short and highly variable cytoplasmic amino and carboxy termini, flanking a first extracellular loop of 53 amino acids and a second shorter loop of 24 amino acids (Amasheh *et al.* 2011). In order to form the TJ barrier complex, the carboxy terminus of claudins binds to

Zonula occludens-1 (ZO1) and to related ZO2 and ZO3 proteins. In gastrointestinal epithelia, TJ regulates the entry of nutrients, ions, and water while restricting pathogen entry. Besides, it is constantly being remodeled when interacts with external stimuli, such as food residues and pathogenic and commensal bacteria (Ulluwishewa *et al.* 2011). Various diseases have been proven to link with claudin mutation. Furuse *et al.* (2002) have reported of claudin-1-deficient mice dying after birth as a result of dehydration. Bacterial endotoxin, lipopolysaccharides (LPSs), or *Clostridium perfringens* enterotoxin (CPE) has been shown to decrease claudin-1, -3, or -4 expression, causing the loss of intestinal TJ barrier function (Sonoda *et al.* 1999, Fujita *et al.* 2012).

In the reproductive tissues, expression of claudin-1, -3, -4, -5, and -7 has been indicated in human endometrium. Generally, claudin-1, -3, -4, and -8 are considered barrier-forming claudins to increase the epithelial tightness, whereas claudin-2 and -7 are pore-forming claudins known to decrease the epithelial tightness. The decreases in claudin-3 and -4 or increases in claudin-2 and -7 expression cause the increases in tissue permeabilities, which have been suggested to associate with ectopic endometriosis and various

endometrial cancer (Gonzalez-Mariscal *et al.* 2003, Pan *et al.* 2007, Gaetje *et al.* 2008). Human immunodeficiency syndrome virus (HIV) employs the defective TJ barrier function through the downregulated *claudin-3* and *-4* mRNA expression and leads to microbial translocation including LPS to initiate mucosal transmission of HIV and HIV disease progression (Nazli *et al.* 2010). Thus, the increased paracellular permeability as reflected by the reduced transepithelial electrical resistance (TER) may associate with the leakiness of TJ and allow normally excluded molecules across the epithelium, resulting in inflammation and pathological conditions in reproductive system or any others.

TJ proteins are regulated by sex steroid hormones, particularly estrogen and progesterone (Satterfield *et al.* 2007, Bailey *et al.* 2010). Isoflavonoids, a class of phytoestrogens mainly found in soy-based foods, which have been evidenced for both estrogenic and anti-estrogenic actions, may modify the TJ barrier expression and function (Brooks & Skafar 2004, Rietjens *et al.* 2013). Genistein (Ge) and daidzein (Di) are the major isoflavones in soybeans, being extensively studied because of their pharmacological and beneficial effects (Wang & Murphy 1994, Banerjee *et al.* 2008). Their effects on the prevention of hormone-dependent breast and prostate cancers are best understood (Barnes *et al.* 1994). Although little is known regarding the effect of soybean isoflavones on TJ barrier function in the endometrium, some studies in the gastrointestinal epithelium have shown the effects of soy isoflavones, especially Ge on the protection of TJs barrier function induced by oxidative stress. Ge has been reported to inhibit the decreased TER and [³H]-mannitol flux as indicators of TJ permeability induced by superoxide anion production in human colonic cell line Caco-2 (Rao *et al.* 2002). The mechanism of Ge action is reported to block the dissociation of TJs, ZO1 and occludins, mediated by tyrosine kinase (Atkinson & Rao 2001). Furthermore, Ge has been reported to block the impairment of TJs barrier function induced by *Salmonella typhimurium* and *Escherichia coli* in Caco-2 cells (Wells *et al.* 1999).

In previous studies, the immortalized porcine glandular endometrial epithelial cell culture (PEG) was characterized and represented as the normal cell culture for examining the effects of Ge and Di on the epithelial function (Palmer *et al.* 2008, Deachapunya & Poonyachoti 2013). Thus, in this study, we aimed to investigate whether the soy isoflavones, Ge or Di, alter the endometrial barrier function in the PEG cells as determined by TER and FITC-dextran flux (FD-4). The mechanism of TER changes by these isoflavones depending on the transcription level of TJs genes in the endometrial tissues, i.e., *claudin-1*, *-3*, *-4*, *-7*, and *-8* and its scaffolds *ZO1* was also examined. The assessment of the ameliorative effect of Ge and Di on the LPS-induced disruption of epithelial barrier protein and function was

finally determined. Understanding the effect of phytoestrogens on endometrial barrier function is critical for uterine health and reproductive success.

Materials and methods

Materials

Ge, Di, insulin, nonessential amino acid, LPS (*E. coli* 0111:B4, purified by phenol extraction), and high purity grade salts were purchased from Sigma Chemical Co. DMEM, Dulbecco's PBS (DPBS), phenol red-free DMEM, fetal bovine serum (FBS), 0.05% trypsin-0.53 mM EDTA, kanamycin, and penicillin-streptomycin were purchased from Gibco BRL. Charcoal-stripped (cs) FBS was purchased from Biowest Co. (Miami, FL, USA).

Cell culture

Immortalized PEG cells were kindly provided by Prof. Scott O'Grady, University of Minnesota. The cells were cultured in DMEM supplemented with 3.7 g/l NaHCO₃, 5% heat-inactivated FBS, 850 nM insulin, 1% non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml kanamycin (standard medium), and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in the air. Before the experiment, the PEG cells were subcultured onto 12 mm diameter transwell insert filters with 0.4 µm membrane pore size. The cell monolayers were fed every 2 days and maintained in the standard media for about 7 days. In an effort to verify the possible regulation of Ge or Di on the epithelial barrier function, the standard cell culture medium was replaced with phenol red-free medium containing 2% csFBS-DMEM and other supplements identical to the standard medium for 2 days and subjected to drug treatment.

Measurement of endometrial TJ barrier function

Porcine endometrial TJ barrier function was evaluated by the measurements of TER and the unidirectional flux of FITC-conjugated dextran (FD-4; average molecular weight 4000) across PEG monolayers in transwell inserts. TER was performed to determine the permeability of TJ lining at the uppermost portion of endometrial epithelial cells. Following the subculture of PEG cells on the transwell inserts, the TER was periodically measured by using EVOM² electrode connected to volt-ohmmeter (World Precision Instruments, Inc., Sarasota, FL, USA) over 24-h intervals before and after drug treatment. To monitor the changes in TER with phytoestrogens and LPS stimulation, the PEG cells grown in 2% csFBS-DMEM for 2 days were measured for TER. After that, Ge or Di (10⁻¹⁰, 10⁻⁸, or 10⁻⁶ M) or their vehicle DMSO were applied to the apical side of cell monolayer and incubated for 48 h. The TER was measured before (0 min) and at 30 min, 1, 2, 24, and 48 h after administration of drugs. Percent changes in TER at each time point from the starting point were calculated and analyzed.

For measurement of paracellular unidirectional flux, FITC-conjugated dextran (FD-4; 1 mg/ml) prepared in

HEPES-buffered salt solution (HBSS): HEPES (25 mM), NaCl (120 mM), KCl (5.4 mM), CaCl_2 (1.8 mM), NaHCO_3 (25 mM), glucose (15 mM) was added to the apical side of the filter-grown PEG monolayers after 48 h treatment with isoflavonoids or LPS. After incubation of FD-4 for 4 h at 37 °C, media samples from the basolateral side were collected and fluorometrically determined at excitation 492 nm and emission 535 nm (Infinite 200 PRO, Tecan, Männedorf, Germany).

LPS stimulation

To examine the effect of Ge and Di on bacterial endotoxin-induced TJ disruption, the PEG cells pretreated with drugs or vehicle for 48 h were replaced with fresh 2% csFBS-DMEM, containing 1 µg/ml of O111:B4 *E. coli* LPSs plus isoflavones and further incubated for 48 h. The measurement of TER was performed before (0 min) and at 30 min, 1, 2, 24, and 48 h after LPS stimulation. The percent changes in TER at each time point of 48-h isoflavones or vehicle treatment before and after LPS stimulation were calculated and compared. The cells from each cell culture insert were then harvested, and the total RNA was extracted for the analysis of TJs mRNA expression.

RT and semiquantitative real-time PCR

To determine the level of mRNA expression, the PEG cells on the cell insert treated with drugs following with or without LPS stimulation for 48 h were subjected to RNA extraction using Trizol (Life Technologies). After precipitation with absolute ethanol, the RNA pellet was collected and diluted with diethylpyrocarbonated-treated water and determined for quantity and quality by spectrophotometry (NanoDrop; Thermo Scientific, Wilmington, DE, USA) at the absorbance wavelength of 260 and 280 nm. RNA samples with a ratio of absorbance at 260/280 nm in the range of 1.8–2.0 were accepted for analysis. Total RNA were reverse-transcribed to the first-strand cDNA using the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories). The protocol was performed according to the manufacturer's instructions. Briefly, total RNA (3 µg) was denatured at 65 °C for 5 min, and then reverse-transcribed in a final volume of 20 µl of the reaction mixture with oligo-(dT)₂₀ primer, iScript reverse transcriptase in the condition of 25 °C for 5 min, 42 °C for 30 min for primer-template hybridization and polymerization, and ended with 85 °C, 5 min for inactivation of the enzyme using the Gradient Thermocycler (Whatman Biometra, Goettingen, Germany). The cDNA product was stored at 4 °C until use. For the real-time PCR analysis, all primers as given in the Table 1 were designed by using The Primer Express Software Version 3.0 (Applied Biosystems) and prepared by Biosearch Technologies (Novato, CA, USA). For semi-quantitative analysis, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was also amplified as an internal control.

To optimize the number of amplification cycles for all examined genes, conventional PCR was performed by the TopTaq Mastermix Kit (Qiagen) for 17–40 rounds using the PEG cells grown in the standard medium according to the manufacturer's manual. The reaction contained TopTaq Master Mix (1.25 units of TopTaq DNA polymerase, TopTaq PCR buffer

Table 1 Primers used for real-time PCR of tight junction genes.

Tight junction genes	Primer sequence (5' → 3')	GenBank accession number
<i>Claudin-1</i>	Fwd: CCCGGTCAATGCCAGATATG Rev: CACCTCCAGAAAGGCAGAGA	NM_001161635.1
<i>Claudin-3</i>	Fwd: CATTATCACAGCGCGGATCA Rev: CGTACACTTTGCATGCTCTG	NM_001160075.1
<i>Claudin-4</i>	Fwd: GTGTAAGGTGCTACCGCTGATTC Rev: AGGGCCATTCTGGAGTCACA	NM_001161637.1
<i>Claudin-7</i>	Fwd: CCATGACTGGAGGCATCATTT Rev: GACAATCTGGTGGCCATACCA	NM_001160076.1
<i>Claudin-8</i>	Fwd: TGGGAAGGACTGTGGATGAAC Rev: AGAGCCAGCAGGGAATCGTA	NM_001161646.1
<i>ZO1</i>	Fwd: AAAAGTGAACACGAGATGCT Rev: AAAGGTGGAGGACTGGAGATGA	NC_010443.4
<i>GAPDH</i>	Fwd: GCA GGT CAG GTC CAC AA Rev: TTC CAC GGC ACA GTC AA	NM_001206359.1

with 1.5 mM MgCl_2 and 200 µM each dNTP) and 400 nM of each primer. The amplification was performed by Gradient Thermocycler (Whatman Biometra) for 45 cycles starting with enzyme activation at 94 °C for 3 min followed by denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and at 72 °C for 1 min for extension followed by 10 min incubation at 72 °C for final extension. The PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide for visualization under u.v light. The amounts of amplicons of all interested genes were linearly increased from 30 to 40 cycles. Therefore, the numbers of amplification cycles were fixed in the 40 cycles. To study the relative quantitative expression of the targeted gene with the housekeeping gene, semiquantitative PCR was performed with Corbett Rotor Gene 6000 (Qiagen) and Evaline EvaGreen E4 Supermix (GeneOn Biotech, Ludwigshafen am Rhein, Germany) containing EvaGreen dye, 400 nM of each primer and 150 nM of the template were mixed in 20-µl reaction. The amplification reaction was performed for 40 cycles, starting with enzyme activation at 95 °C for 3 min following with denaturation (95 °C, 30 s), annealing (60 °C, 30 s), and extension (72 °C, 30 s). The interested gene expression was analyzed using the relative method ($2^{-(1 \times \Delta C_T)}$), which normalized the threshold cycle (C_T) of an interested gene to an internal control gene (porcine *GAPDH*). The relative fold changes in the interested gene expression in response to drug treatment were analyzed using the relative comparative method ($2^{-(1 \times \Delta \Delta C_T)}$), which calculated the fold changes in the interested gene expression in drug-treated group or LPS-inoculated group from the control DMSO group.

Statistical analyses

All values are presented as mean $\pm$ S.E.M., and *n* is the number of cell monolayers from at least three different cell culture passages. Statistical analyses were performed using Prism 5.0, GraphPad Software, Inc., San Diego, CA, USA, the differences between the control and experimental means were analyzed using a Student's *t*-test or ANOVA where appropriate. *Post-hoc* test was additionally performed with the Dunnett's test to compare the differences from control or the Newman-Keuls test to compare the differences between two groups. A *P* value < 0.05 was considered to be significant.

Results

Effect of Ge and Di on TER

In the immortalized PEG cells, the baseline values of TER across the cell monolayer before addition of each drug were $1664 \pm 136.0 \Omega \times \text{cm}^2$ ($n=18$). In all experiments, the percent change in TER from the baseline value (% change in TER from an initial value of time 0) was calculated for drug treatment at each time point. Under control conditions, after replacing with the newly tested medium, the percent change in TER increased by 30% from the initial value in 30 min, gradually decreased to the baseline TER within 24 h, and remained unchanged until 48 h of the observation period (Fig. 1). In the presence of Ge or Di in the culture medium, the percent change in TER was variable as shown in Fig. 1A and B respectively. The TER value of cells treated with Ge at

Figure 1 Effects of genistein (A) and daidzein (B) on the percent change in transepithelial resistance (TER) in the PEG cells. The cells were treated for 48 h with genistein or daidzein at 10^{-10} (closed down triangle), 10^{-8} (closed diamond) or 10^{-6} M (closed circle) or vehicle control DMSO (open square). The TER was measured at 30 min, 1, 2, 24, and 48 h following drug treatment. Values are the mean $\pm$ S.E.M. ($n=6$ in each group). * $P<0.05$ or ** $P<0.01$ denotes difference from control at each time point.

low concentration (10^{-10} M) slightly decreased by 4% from the initial value during the first 30 min. The value then remained unchanged for 2 h before decreasing by 23% at 24 and 48 h, which was significantly lower than the TER changes observed in the control group. Higher concentration of Ge (10^{-8} or 10^{-6} M) showed the profile change in TER during 30 min to 24 h, similar to the control group, while a significant increase in the TER was observed following 48-h incubation period compared with the control group (Fig. 1A).

Similarly, treatment with Di at the lowest concentration (10^{-10} M) significantly decreased the percent change in TER by 20–30% during 2–24 h (Fig. 1B). Nevertheless, the increase in the TER was found after 2 h incubation with the highest concentration of Di used in this study (10^{-6} M) (Fig. 1B).

Effect of Ge and Di on TER after exposure to LPS

As LPS has been shown to impair the epithelial barrier function in intestinal Caco-2 cell study (Wells *et al.* 1993), we further clarified the modulatory effects of soy isoflavones on the LPS-induced epithelial barrier disruption in the endometrial cells. After pretreatment of the PEG cells with Ge or Di for 48 h, the TER were continually determined following 30 min, 1, 2, 24, and 48 h in the presence of LPS and each compound. As shown in Fig. 2, cell exposure to LPS alone (1 $\mu\text{g}/\text{ml}$) increased the percent change in TER by 20–30% during 30 min to 2 h and subsequently decreased the TER by 30% at 24 and 48 h. Following 2 h with LPS stimulation, Ge at 10^{-10} M did not affect the TER, but a significant reduction in the TER was observed with 10^{-8} or 10^{-10} M Ge treatment as compared with the untreated LPS group. During 24–48 h, the TER of all Ge-treated groups were maintained, which was significantly higher than those of untreated LPS groups (Fig. 2A). Similar to Ge, a pattern of the percent change in TER after LPS stimulation was also observed in Di treatment groups with different concentration responses (Fig. 2B). With LPS stimulation for 24–48 h, the control LPS group continued to decrease the TER lower than the value before LPS stimulation, whereas the TER was restored in all groups receiving either Di or Ge treatment (Fig. 2A and B).

Effect of Ge and Di on unidirectional FITC-dextran flux

To further assess the effect of isoflavones on paracellular permeability, FITC FD-4, which is commonly used as a marker of paracellular transport of macromolecules, was additionally performed. As shown in Fig. 3A, pretreatment with Ge or Di for 48 h had no effect on the apical-to-basolateral FD-4 flux as compared with the control DMSO. Moreover, the FD-4 flux was not affected after continuous culture of cells with LPS alone. However,

Figure 2 Effects of genistein (A) and daidzein (B) pretreatment on the percent change in transepithelial resistance (TER) in response to lipopolysaccharides (LPS) stimulation in the PEG cells. The cells were incubated with 1 $\mu\text{g/ml}$ of LPS for 48 h after pretreatment with genistein or daidzein at 10^{-10} (closed down triangle), 10^{-8} (closed diamond) or 10^{-6} M (closed circle) or vehicle control DMSO (open square). The TER was measured at 30 min, 1, 2, 24, and 48 h after LPS stimulation. Values are the mean $\pm$ S.E.M. ($n=6$ in each group). * $P<0.05$ or ** $P<0.01$ denotes difference from control at each time point.

treatment with Ge at 10^{-8} or 10^{-6} M or Di at 10^{-10} or 10^{-6} M in the presence of LPS significantly decreased the FD-4 flux, although not in a dose-dependent manner.

Effect of Ge and Di on TJ gene expression

To evaluate the genomic effects of Ge and Di on TJ gene expression, which are important components of cellular TJs, total RNA of the PEG cells treated with these isoflavones for 48 h were analyzed by quantitative real-time RT-PCR using primers specific to different TJ genes as appeared in Table 1. Without LPS stimulation, the untreated PEG cells expressed all six TJ genes *claudin-1*, *-3*, *-4*, *-7*, *-8*, and *ZO1* and about one- to 1.5-fold of housekeeping gene *GAPDH* as shown in Fig. 4. Treatment with 10^{-8} M or 10^{-6} M Ge revealed a twofold increase in *claudin-3* mRNA whereas Ge only at 10^{-6} M caused a 1.5-fold increase in *claudin-4* mRNA compared with the corresponding control

(Fig. 5A). In addition, Di at 10^{-6} M or 10^{-8} M increased *claudin-8* mRNA expression by twofolds (Fig. 5B, $P<0.05$). All concentrations of Ge and Di used in this study did not significantly alter the *claudin-1*, *-7*, and *ZO1* mRNA expression.

Effect of Ge and Di on TJ gene expression after exposure to LPS

Even though most of the TJ genes being studied were not affected directly by these soy isoflavones, it was worth of studying the modulatory effects of these compounds on the expression of TJ genes in the PEG cells exposed to LPS. Following inoculation with LPS (1 $\mu\text{g/ml}$) for 48 h, the cells significantly increased the mRNA expression of *claudin-7* and *ZO1*, but decreased the *claudin-8* mRNA expression when compared with the cells without LPS exposure (Fig. 4). Treatment of the PEG cells with Ge at

Figure 3 Effects of genistein and daidzein pretreatment on unidirectional FITC-dextran flux (FD-4) in the PEG cells before (A) and after (B) lipopolysaccharides (LPS) stimulation. FD-4 flux was measured across PEG monolayers after incubation with genistein or daidzein at 10^{-10} , 10^{-8} or 10^{-6} M or vehicle control DMSO without LPS for 48 h (A) followed by incubation with drugs with LPS for 48 h (B). Values are expressed as means $\pm$ S.E.M. ($n=4$ experiments in each group). * $P<0.05$ or ** $P<0.01$ denotes difference from corresponding control group DMSO (A) or DMSO+LPS (B).

Figure 4 Endogenous mRNA expression tight junction genes *claudin-1*, *-3*, *-4*, *-7*, *-8*, *ZO1* normalized to housekeeping gene *GAPDH* in the PEG cells. The cells were cultured without (no LPS) or with 1 µg/ml of LPS (+LPS) for 48 h. Total RNA was analyzed by real-time RT-PCR. Values are the mean $\pm$ S.E.M. ($n=6$ in each group). * $P<0.05$ or ** $P<0.01$ denotes difference between no LPS and +LPS groups.

10^{-6} M or Di at 10^{-8} M or 10^{-6} M was found to restore the decreased *claudin-8* mRNA expression being affected by LPS (Fig. 6). Ge at 10^{-6} M also promoted the *claudin-4* and/or *-7* mRNA expressions in the LPS-treated cells (Fig. 6A). Treatment with high and medium concentrations of Di (10^{-8} M or 10^{-6} M) significantly increased by two- to sixfold the expressions of all examined claudins. However, upregulated *ZO1* expression in the LPS-treated cells was only found with Di treatment at 10^{-8} M.

Discussion

Epithelial barrier function has a crucial role in health and disease. Destruction of epithelial barrier integrity caused by alterations in TJ protein structure and function followed by permeation of microorganism from the lumen can induce excessive activation of the mucosal immune system causing tissue damage. Indeed, increases in endometrial permeability are usually found in various reproductive diseases, chronic inflammation, and cancer in particular (Förster 2008). Soybean isoflavones with chemical structure and function similar to the endogenous estrogen have been reported to exhibit ameliorative effects on some of these diseases, although the precise underlying mechanisms remain to be clarified. Ge, Di, and their respective β -glucoside forms are the primary isoflavones in soybeans and exert higher potency than other flavonoids (Messina 2002). In the previous study using human intestinal Caco-2 cells, Di and other flavonoids hesperetin, naringenin, and morin that have a different hydroxyl group on A-ring structure from the other flavonoids show the enhancing effect on the barrier integrity, while Ge has the neutralized effect (Noda *et al.* 2012).

In this study, the aglycone Ge and Di were used because the presence of these two aglycones (unconjugated form) was found to be higher in soy food than β -glycosides (conjugated form) (Setchell 1998). Moreover, only unconjugated form exerts estrogenic effects on the target organs. Even though in the intestinal tissues and liver the isoflavones are metabolized to the compounds with lesser effect, the plasma concentration of total flavonoid derivatives ranging from 0 to 4 µM is equivalent to an intake of 50 mg aglycone (Manach *et al.* 2005). The plasma aglycone will reach and accumulate in the target organs including uterus to produce many biological effects (Chang *et al.* 2000). Recent study by Bitto *et al.* (2010) has revealed that an ingestion of Ge at a dose of 54 mg/day is an effective suppression dose in the treatment of endometrial hyperplasia. As has been reported in many observations that Ge in particular usually shows biphasic effects depending on

Figure 5 Effects of genistein (A) and daidzein (B) on the relative fold change in mRNA expression of tight junction genes *claudin-1*, *-3*, *-4*, *-7*, *-8*, *ZO1* in the PEG cells. The cells were treated for 48 h with genistein or daidzein at 10^{-10} , 10^{-8} or 10^{-6} M or vehicle control DMSO. Total RNA was analyzed by real-time RT-PCR. Values are the mean $\pm$ S.E.M. ($n=6$ in each group). Scale bars with different letters (a, b) mean significantly different at P value <0.05 .

Figure 6 Effects of genistein (A) and daidzein (B) on the relative fold change in mRNA expression of tight junction genes *claudin-1*, *-3*, *-4*, *-7*, *-8*, *ZO1* in the PEG cells. The cells were incubated with 1 μ g/ml of LPS for 48 h after pretreatment with genistein or daidzein at the 10^{-10} , 10^{-8} or 10^{-6} M or vehicle control DMSO. Total RNA was analyzed by real-time RT-PCR. Values are the mean $\pm$ S.E.M. ($n=6$ in each group). Scale bars with different letters (a, b) mean significantly different at P values <0.05 .

concentrations, we thus investigated the soy isoflavone effects by using 10^{-10} M, 10^{-8} M, or 10^{-6} M as low, medium, or high concentration (Polkowski & Mazurek 2000). In addition, the isoflavone effects on TER at the varied time were also focused as its effects have been suggested to be mediated by many pathways depending on time courses, acute vs long-term effects.

This study demonstrated that Ge and Di at specific concentrations affected the barrier function by alterations in TJ permeability in immortalized glandular epithelial monolayer, which have previously represented as an effective model for evaluating the effect of phytoestrogen on electrolyte transport (Deachapunya & Poonyachoti 2013). The underlying mechanism of these effects was thought to be relevant to alterations in TJ proteins, claudins, and ZO expression, as the genomic effects of these two isoflavonoids have been reported to affect the transcription level of these TJs proteins in intestinal epithelial cells (Suzuki & Hara 2009).

In the early part of this study, the periodical changes in endometrial TER under vehicle control condition revealed that the percent change in TER began to increase within 30 min, gradually decreased close to the baseline at 24 h, and maintained up to 48 h. This suggests that it requires at least 24 h to obtain a stable TER after replacement of cells with fresh media. Treatment with high concentration of Ge (10^{-8} M or 10^{-6} M) did not change the TER profile within the first 24 h, but it significantly increased the TER at 48 h compared with the control. However, low concentration of Ge (10^{-10} M) appeared to slightly decrease the TER at 30 min and markedly decrease at 24–48 h. This biphasic effect of Ge has been evidenced for studying biological activities in normal and cancer cells (Polkowski & Mazurek 2000). For example, in breast cancer cells, Ge at high concentration ($>10^{-5}$ M) inhibits growth of MCF-7 cells, whereas Ge as low as 10^{-6} or 10^{-8} M stimulates cell growth through the activity of estrogen receptor alpha (ER α) (Wang *et al.* 1996). In addition, Ge at the nanomolar range appears to elicit estrogen-like proliferative effects (Martin *et al.* 1978), but reduces tumor cell proliferation (Dixon-Shanies & Shaikh 1999) and blocks angiogenesis at the micromolar concentration (Fotsis *et al.* 1993). Although the underlying mechanisms remain to be explored, the biphasic effect of Ge on the TJ permeability could be attributed to different signaling pathways responsible for different responses. A number of studies have shown that activation of classical ERs by endogenous ligand can disrupt the cytoskeletal architecture, leading to increased endothelial cell permeability (Groten *et al.* 2005, Aberdeen *et al.* 2008). On the other hand, high concentration of Ge produces multiple intracellular effects, including inhibition of tyrosine-specific protein kinases (Akiyama *et al.* 1987, Akiyama & Ogawara 1991) and topoisomerase II (Markovits *et al.* 1989). Taken together, it raises possibility that, in this study, high concentration of Ge enhances TJ barrier function through an inhibition of tyrosine kinase, whereas the low concentration of Ge reduces the barrier function through the ER pathway. However, the precise mechanism requires further study.

In addition, the decreased TER within 30 min–2 h after isoflavones treatment should not be explained by their genomic effects. This observation may correlate with our previous study demonstrating an acute effect of Ge on stimulation of chloride secretion in endometrial cells (Deachapunya & Poonyachoti 2013). Thus, the reduction in transcellular resistance caused by the opening of chloride ion-conductive pathway may account for the Ge-induced decrease in TER, albeit the TER is mainly dependent on the paracellular pathway (Madara *et al.* 1988). The increased transepithelial electrolyte transport, providing the driving force for paracellular transport so-called the solvent drag, may be associated.

However, a reduction in TER in response to low concentration of Ge or Di is unlikely due to its

cytotoxicity. The cell viability as assessed by trypan blue exclusion increased by 20–30% from initial after 48-h incubation with these isoflavones (observed data). The decreased TER is known to cause the permeation of pro-inflammatory molecules from the lumen, leading to the excessive activation of the mucosal immune system. The immunological responses involve many cytokines and cell-mediated processes, resulting in sustained inflammation and tissue damage. Our results suggest that the soy isoflavonoid-mediated regulation of the endometrial barrier may be partially associated with their biological effects on reproductive organs. A defect in endometrial barrier is involved in several diseases, such as chronic endometritis, endometriosis, or cancer (Martin & Jiang 2009).

Apart from a weak estrogenic effect by binding to ERs (Kuiper *et al.* 1997, Kim *et al.* 1998), the mechanism of Ge and Di action has been indicated as inhibitors of tyrosine kinase, although Di is less potent (Akiyama *et al.* 1987). Since our recent study using the PEG cells that demonstrated the Ge stimulation of anion secretion via a tyrosine kinase-dependent pathway (Deachapunya & Poonyachoti 2013), it is possible that the tyrosine kinase-mediated phosphorylation of TJ protein is relevant to Ge effect on regulating TJs expression and function. The phosphorylation status of TJ protein has been suggested to determine TJs structure and function (Rao *et al.* 1997).

It has been reported that impairment of TJ barrier function is primarily caused by pathogenic bacteria (Ulluwishewa *et al.* 2011), inflammatory cytokines (Youakim & Ahdieh 1999, Ahdieh *et al.* 2001), and reactive oxygen species (Basuroy *et al.* 2003). The underlying mechanism has been proposed to be mediated through the tyrosine kinase inducing the disassembly of TJ protein complex (Suzuki & Hara 2009). Therefore, the protective effect of Ge and Di on the decreased endometrial barrier function was continuously examined by challenging PEG cells with bacterial endotoxin O111:B4 *E. coli* LPSs following 48 h pretreatment with Ge, Di, or vehicle. The bovine endometrial epithelial and stromal cells have been reported to express TLR4 and MyD88 protein, which binds to LPS and stimulates the cellular release of prostaglandins, cytokines, and chemokines that coordinate the immune and physiological responses including alteration of tissue permeability (Davies *et al.* 2008, Herath *et al.* 2009). The expression of toll-like receptors has been identified in the porcine endometrium, although the regulation of TJ barrier function by microorganism has never been indicated (Croy *et al.* 2009, Østrup *et al.* 2010). In this study, challenging the PEG cells with LPS significantly decreased TER after 24 h. This finding was consistent with reports of other studies in endometrial cells (He *et al.* 2009) or intestinal cell monolayers (Chen *et al.* 2004). Pretreatment with Ge or Di for 48 h before challenging with LPS inhibited the decreased TER

induced by LPS. This evidence suggests the potential effect of these soy isoflavones on prevention of the disrupted TJ barrier function induced by pathogens.

Besides the TER, the effects of Ge and Di on the unidirectional flux of FD-4 with a molecular weight of 4 kDa were further tested for macromolecules transport across the leaky epithelium. Unlike the TER, this FD-4 flux across the PEG cells was not responsive to LPS or both isoflavones pretreatment. However, in the presence of LPS, Ge (10^{-8} and 10^{-6} M) or Di (10^{-10} and 10^{-6} M) was found to promote strengthening of PEG as indicated by the increased TER and decreased FD-4 flux. Similar to our study, the permeability of TJ barrier function as assessed by the TER measurement that was not coordinated with the FD-4 flux was previously evidenced in the study of flavonoids in Caco-2 colonic epithelia (Noda *et al.* 2012). They discussed that the TJ integrity and permeability of Caco-2 colonic epithelia are coordinately regulated by several TJ proteins, in which alterations in protein composition thereby create the variation in paracellular barrier to ions and solutes. TJ proteins indeed have barrier- or pore-forming properties, which allow the paracellular transport of the selected molecules based on charge- and/or size. Many claudins, i.e. claudin-4 and -8, have been characterized as a sodium barrier-formers leading to increased TER but not to macromolecules such as 4Kd FITC-dextran. The different paracellular pathways via the other family of TJ protein occludin, tricellulin, and marvelD3 have been postulated for transport of macromolecules (Raleigh *et al.* 2010). The paracellular pathway that is permeable to macromolecules has been identified as a 'leak pathway' which is unknown to date, but can be determined by FITC-dextran permeability assay (Krug *et al.* 2014).

In porcine uterine tissues, the mRNA expression of TJ proteins has been detected (Bailey *et al.* 2010, Samborski *et al.* 2013). The expression of claudin-1, -2, -3 and -4 proteins are dynamic through the estrous cycle or pregnancy under the regulation of ovarian sex steroids and placental factors, i.e., estrogen, progesterone, and prolactin (Bailey *et al.* 2010). We, therefore, further studied whether the changes in TER may be relevant to the genomic effects of Ge and Di on the transcription level of claudin and ZO1. The effects of isoflavones on the expression of occludin were not included in this study as it was not affected by LPS (Youakim & Ahdieh 1999, Al-Sadi & Ma 2007). Moreover, occludin depletion or knockout did not affect the barrier function of TJs both *in vivo* and *in vitro* studies (Schulzke *et al.* 2005, Xia *et al.* 2005). This study was the first to detect the expression of TJ mRNA, the *claudin-1*, -3, -4, -7, or -8, and *ZO1* at a higher level than the housekeeping *GAPDH* in glandular endometrial epithelial cells, with or without LPS inoculation.

Treatment with Ge or Di at 10^{-6} M for 48 h was found to upregulate *claudin-3* and -4 or -8, respectively,

suggesting that this effect was mediated via a genomic pathway. Other study has shown the upregulation of claudin-4 but not claudin-3 in surface and glandular epithelia in response to progesterone level (Bailey *et al.* 2010). As Ge and Di has an affinity for ERs to produce an estrogenic activity, these isoflavones may regulate transcription of ER-target genes via the estrogen-responsive elements (EREs) present within the gene promoter regions. Even though, *claudin-3* and *-4* or *-8* genes have not been well characterized, our results imply that claudin-3 and *-4* or *-8* may be the gene target of Ge or Di respectively. It is known that Ge is relatively more potent than Di and binds to ER β with much higher affinity than ER α (Zava & Duwe 1997). Thus, the different target genes for Ge and Di action could be mediated by different ER subtypes or other yet unknown mechanisms.

Highly claudin-3 and *-4* expressed epithelia not only increase TJ defense function, but also induce cell-to-cell adhesion, as has been suggested in atypical hyperplasia and endometroid adenocarcinoma (Pan *et al.* 2007, Konecny *et al.* 2008). In addition, claudin-3 and *-4* are homologous to CPE receptor (CPE-R) in the intestine, which is capable for CPE binding and causing cell cytotoxicity (Long *et al.* 2001). Accordingly, long-term treatment with high concentration of Ge should be taken into consideration and needs to be examined, as it may transform the endometrium to endometroid cancerous cells and susceptible to CPE in human and animals.

Although TJ protein composition is known to correlate with barrier function or tissue permeability, our results revealed that *claudin-8* gene expression was increased by Di (10^{-8} and 10^{-6} M) at 48 h without any difference in the TER. This evidence could be explained by the fact that the expression of several types of claudin proteins seems to be more important than only one type like claudin-8 expression. For an example, distal tubule expressing claudin-1, *-3*, and *-8* reveals lower electrical resistance than the collecting segment expressing claudin-1, *-3*, *-4*, and *-8* even though they both expressed the claudins-8 (Gonzalez-Mariscal *et al.* 2003). These findings support ours that why the increased claudin-8 expression induced by Di 10^{-8} M did not change the TER as compared with the control which expressed claudins-1, *-3*, *-4*, and *-8*. Nevertheless, the previous study by Amasheh *et al.* (2009) suggested that increased claudin-8 levels have been shown to promote the sealing of the paracellular barrier.

However, the significance of claudin-3 and *-4* expression for the tightening of endometrium could not be dismissed. The Ge (10^{-8} M or 10^{-6} M) induced increases in claudin-3 and *-4* expressions, but not claudin-8, were shown to be parallel with the increased TER. This finding was supported by the study of endometrium of HIV and endometriosis patients showing that the leakiness of endometrium is due to the result

of the decreased claudin-3 or/and *-4* expression (Pan *et al.* 2007, Gaetje *et al.* 2008, Nazli *et al.* 2010).

In the PEG cells challenged with LPS, a reduction in TER after 48 h was correlated with downregulation of *claudin-8* mRNA expression and upregulation of *claudin-7* and *ZO1*. The LPS effects on the TJ protein expression have been reported to be mediated through the signaling pathway, resulting in the release of TNF α , IL8, and prostaglandin in cultured bovine endometrial epithelial cells (Davies *et al.* 2008, Herath *et al.* 2009). In respiratory epithelia, the release of TNF α and IL8, which is mediated by NF- κ B, p38MAPK, and ERK1/2 MAPK, has been indicated to relate with the decreased expression of some TJ proteins, i.e. claudin-5 and *ZO1*, causing an increase in epithelial permeability (Chow *et al.* 2010). This similar mechanism may apply to the downregulation of claudin-8 by LPS in the PEG cells. Treatment with Ge or Di was found to restore the downregulated *claudin-8* induced by LPS. Even though the underlying mechanism was not investigated in our study, we speculate that Ge or daidzein, well-known inhibitors of protein kinase activity (Akiyama *et al.* 1987), may inhibit the downstream signaling of NF- κ B and thereby restore the LPS downregulated *claudin-8*.

Interestingly, Ge at all concentrations suppressed the LPS-induced decrease in TER at 48 h, but none of examined TJ gene expression was increased by Ge 10^{-8} M in LPS-inoculated cells (Fig. 6A). Even though Ge 10^{-8} M could not alter the mRNA expression of barrier-forming claudin-1, *-3*, *-4*, *-8*, or *ZO1*, the other claudin, i.e. claudin-2, which is known as pore-forming claudin generally presented in small intestinal leaky epithelia but not in kidney or endometrium could be induced by LPS. Unfortunately, claudin-2 have low expression and have not been reported as the target of LPS in porcine endometrium. However, the suppressive effects of Ge 10^{-8} M on the LPS-induced claudin-2 should be concerned.

In addition to the alteration of TJs protein expression and function, the alteration of other protein such as myosin light-chain kinase (MLCK) has been proposed to mediate the effect of LPS on decreased TER. It is possible that Ge 10^{-8} M may reverse the LPS-induced the decrease in TER through MLCK-dependent pathway. Similar explanations could be applied to the effects of Ge or Di on the increased expression of examined TJ genes during LPS inoculation; however, the exact mechanisms remain to be elucidated.

In conclusion, our results demonstrated that the soy isoflavones Ge and Di influences the TJ protein and function in both constitutive and pathogens-activated conditions in endometrial epithelial cells. High concentration (10^{-8} to 10^{-6} M) of these isoflavones was effective to reveal the promotive effect on the TJ protein expression and barrier function. LPS induced the disruption of the endometrial barrier by the upregulation

of *claudin-7* and ZO1 and downregulation of *claudin-8*. The impairment of TJ barrier function induced by LPS was ameliorated by pretreatment with soy isoflavones. The possible mechanism of soy isoflavones may be associated with their promotive effects on the *claudin-1*, *-3*, *-4*, *-7*, *-8*, and ZO1 expression. Thus, the use of soybean isoflavones to restore the barrier function may be a new aspect of the therapy of epithelia weakness during bacterial infection or endotoxemia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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