รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัยเรื่อง

ฤทธิ์ระยะยาวของสารเจนีสเทอีนต่อการแสดงออกของยีนและโปรตีนขนส่งไอออนในเซลล์เยื่อบุมดลูกเพาะเลี้ยงชนิดไม่ตาย

Long term effect of genistein on gene and protein expression of ion transporter in immortalized endometrial epithelial cells

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แหล่งทุนสนับสนุนงานวิจัย
งบประมาณเงินรายได้มหาวิทยาลัยศรีนครินทรวิโรฒ
ประจำปีงบประมาณ 2553

CONTENT

บทคัดย่อ

เจนิสทีอีนเป็นสารไฟโตเอสโตรเจนที่ออกฤทธิ์เฉียบพลันกระตุ้นการหลั่งคลอไรด์ในเซลล์เยื่อบุมดลูก เป็นที่น่าสนใจว่าเจนีสทีอีนจะมีผลระยะยาวในการเปลี่ยนแปลงการขนส่งไอออนตลอดจนปรับเปลี่ยนการแสดงออกของยีนที่ควบคุมการขนส่งไอออน ดังนั้นจึงได้ทำการศึกษาฤทธิ์ระยะยาวของเจนีสทีอีนต่อการเปลี่ยนแปลง membrane permeability และการหลั่งคลอไรด์ รวมถึงการแสดงออกของยีนของโปรตีนขนส่งที่เกี่ยวข้องกับการหลั่งคลอไรด์ ได้แก่ small-conductance Ca^{2+}-activated K^{+} channel (SK) และ cystic fibrosis transmembrane conductance regulator (CFTR) ในเซลล์เยื่อบุมดลูกสุกร โดยทำการศึกษาในเซลล์เยื่อบุมดลูกสุกรเพาะเลี้ยงชนิดไม่ตายที่เลี้ยงในภาวะปราศจากเอสโตรเจนและได้รับเจนิสทีอีนขนาด 1, 10, 20 μM เป็นเวลา 4 วัน ผลการศึกษาพบว่าเซลล์กลุ่มควบคุมในภาวะพร่องเอสโตรเจนและกลุ่มที่ได้รับเจนีสทีอีนขนาด 1 μM มีค่า transepithelial resistance (TER) ลดลงในวันที่ 4 เมื่อเปรียบเทียบกับวันที่เริ่มทดลอง (day 0) ขณะที่ไม่พบการเปลี่ยนแปลงค่า TER ในกลุ่มที่ได้รับเจนิสทีอีน 10 และ 20 μM เมื่อนำเซลล์มาติดตั้งบน Ussing chamber พบว่าเซลล์ที่ได้รับเจนิสทีอีน 20 μM เป็นเวลา 4 วัน มีค่า basal potential difference และ resistance สูงขึ้น แต่ลดการหลั่งคลอไรด์ที่ถูกกระตุ้นโดย forskolin (1 μM) เมื่อเปรียบเทียบกับกลุ่มควบคุม ในทางตรงข้ามเซลล์ที่ได้รับเจนิสทีอีน 1 μM กระตุ้นการหลั่งคลอไรด์ที่ถูกกระตุ้นโดย uridine triphosphate (5 μM) และเซลล์ที่ได้รับเจนิสทีอีนทุกความเข้มข้นไม่มีผลเปลี่ยนแปลงการหลั่งคลอไรด์ที่ถูกกระตุ้นโดย prostaglandin E_2 (3 μM) ผล RT-PCR พบการแสดงออกของ SK1, SK2, SK3 และ CFTR mRNA การให้เจนีสทีอีนขนาด 10 μM เป็นเวลา 48 ชั่วโมง เพิ่มการแสดงออกของ SK1, SK2 และ CFTR mRNA ในทางตรงข้าม เจนีสทีอีน 20 μM ลดการแสดงออกของ SK3 และ CFTR mRNA ผลการทดลองแสดงให้เห็นว่าการให้เจนีสทีอีนระยะยาวมีผลรักษา membrane permeability และควบคุมการหลั่งคลอไรด์ที่ถูกกระตุ้นผ่าน cAMP และ Ca^{2+} นอกจากนี้เจนีสทิอีนยังมีผลปรับเปลี่ยนการแสดงออกของ SK และ CFTR mRNA โดยขึ้นอยู่กับความเข้มข้นของเจนีสทิอีน ผลดังกล่าวจะเป็นข้อมูลสำคัญในการพิจารณาประยุกต์ใช้สารไฟโตเอสโตรเจนเจนีสทิอีนในการปรับเปลี่ยนการทำงานของเซลล์เยื่อบุที่มีการขับหลั่งสารน้ำและอิเล็กโทรไลต์ผิดปกติในระยะยาว

ABSTRACT

Genistein has been previously shown to have an acute effect on stimulating Cl^- secretion in porcine endometrial epithelial cells. It is of interest to investigate the long-term effect of genistein on modulation of membrane permeability, Cl^- secretion and expression of transport-related genes involving Cl^- secretion, i.e. small-conductance Ca^{2+}-activated K^+ channel (SK) and cystic fibrosis transmembrane conductance regulator (CFTR), in the porcine endometrial epithelial monolayers. The experiments were performed using immortalized porcine endometrial epithelial cells grown in estrogen-free media in the absence or presence of genistein 1, 10 and 20 μM for 4 days. Transepithelial resistance (TER) as measured by voltohmmeter was significantly reduced on day 4 compared to day 0 in vehicle control or 1 μM genistein-treated groups whereas such a reduction was prevented in the presence of genistein 10 or 20 μM. Experiments with Ussing chamber exhibited an increase in the basal potential difference and resistance but a decrease in Cl^- secretion induced by forskolin in cells treated with genistein 20 μM. While all genistein treatment groups having no effect on the PGE_2-induced increase in Cl^- secretion, only genistein 1 μM significantly increased the Cl^- secretion in response to UTP. RT-PCR experiments demonstrated the mRNA expression of SK1, SK2, SK3 and CFTR. Treatment of monolayers with genistein 10 μM for 48 hrs increased SK1, SK2 and CFTR mRNA whereas genistein 20 μM decreased the SK3 and CFTR expression. The results indicated the long term treatment of genistein on maintenance of membrane permeability and Cl^- secretion mediated by intracellular cAMP and Ca^{2+}. The expression of SK and CFTR was concentration-dependently modulated by the genomic action of genistein. The present results may be of benefit for therapeutic application of genistein in the long term treatment of electrolyte transport disorders in epithelia.

INTRODUCTION

Genistein is an isoflavone phytoestrogen mostly present in leguminous plants, especially soybean and its products, including soy flour, soy milk and tofu (Cederroth and Nef, 2009). It is structurally related to estrogen and has been reported to interact with estrogen receptor and exert estrogenic activity. Genistein and other phytoestrogens are increasingly used as hormone replacement as they have potential health benefits in the treatment and prevention of estrogen-related diseases such as cardiovascular disease and osteoporosis, and the reduction of the risk of breast cancer and endometrial cancer (Anderson et al., 1999; Goldwyn et al., 2000). In addition, genistein has been proposed as a promising therapeutic for cystic fibrosis, through its modulation of transport proteins involved in the Cl^- secretion (Andersson et al., 2003, Schmidt et al., 2007).

Genistein has been shown to regulate ion transport in both native epithelia and cultured epithelial cells. It has been demonstrated to stimulate Cl^- secretion in the rat colon, mouse intestine, human colonic cell line and airway epithelia (Diener and Hug, 1996; Illek et al., 1996; Mall et al., 2000; Baker and Hamilton, 2004). Recently, genistein was also shown to activate Cl^- secretion in endometrial epithelial cells (Deachapunya and Poonyachoti, 2009; 2010). A number of transporter proteins involved in Cl^- secretion including the cystic fibrosis transmembrane conductance regulator (CFTR), K^+ channels, and Na^+-K^+-$2Cl^-$ cotransporter have been modulated by genistein (Diener and Hug, 1996; Illek et al., 1996; Niisato et al., 1999). Most studies concern the acute effect of genistein; however, little is known of the long-term effect of genistein on the transport-related activity.

Several cellular mechanisms have been proposed for the action of genistein, including a direct activation of CFTR (Weinreich et al., 1997), modulation of CFTR by tyrosine kinases (French et al., 1997), protein phosphatases (Illek et al., 1998) or protein

kinases (Sears et al., 1995). The direct action of genistein on CFTR is suggested from patch-clamp studies of heterologously expressed CFTR which reports that genistein increases the activity of CFTR by interaction with CFTR at nuclear binding domain 2 (French et al., 1997; Weinreich et al., 1997). However, the role of genistein on the phosphorylation and dephosphorylation-dependent regulation of Cl^- secretion is not well defined and seems to be tissue or cell-specific. Recently, genistein activates Cl^- secretion via inhibition of a phosphodiesterase 3-dependent pathway in mouse jejunum (Chao and Hamiton, 2009).

Endometrial epithelial cells play an important role on regulation of electrolyte and fluid volume and composition within uterine cavity, providing an optimal intrauterine environment for reproductive processes. The transport-related activities of the surface and glandular epithelial cells have been shown to be regulated by several hormones, growth factors, cytokines and a number of signaling molecules. Electrophysiological studies of cultured endometrial epithelial cells has recently demonstrated an acute stimulatory effect of genistein on Cl^- secretion via activating apical Cl^- channel and basolateral K^+ channel together with bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter (Deachapunya and Poonyachoti, 2010). Since genistein interacts with estrogen receptor, it is of interest whether the long term uses of genistein modulate the electrolyte transport across the endometrial epithelium. Thus the aim of this study was to investigate the long term effect of genistein on ion transport function and the gene expression of small-conductance Ca^{2+}-activated K^+ channel and CFTR in immortalized porcine endometrial epithelial cell. These cells express estrogen receptors both alpha and beta subtypes (Deachapunya and Sutthasinee, 1996), providing a suitable model for studying the transport-related properties of estrogen-like substances.

MATERIALS AND METHODS

Materials

Genistein, forskolin, uridine-5'-triphosphate (UTP), prostaglandin E_2, insulin, non-essential amino acid, L-glutamine and high purity grade salts were purchased from Sigma Chemical Co (St. Louis, MO). Dulbecco modified Eagle's medium (DMEM), phenol red-free DMEM, fetal bovine serum (FBS), charcoal-stripped FBS, 0.05% trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA), kanamycin, and penicillin-streptomycin were purchased from Gibco (Grand Island, NY).

Cell culture

Immortalized porcine endometrial epithelial cells (PEG cells) were kindly provided by Professor Scott O'Grady, University of Minnesota. The PEG cells were primary endometrial epithelial cells that were stably transfected with the catalytic subunit of human telomerase. These cells showed phenotypic and functional characteristics as the primary epithelial cells (Palmer-Densmore et al., 2005). The PEG cell passage number 62-80 was cultured in DMEM supplemented with 3.7 g/L $NaHCO_3$, 10% heat-inactivated FBS, 5 μg/ml insulin, 1% non-essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin (standard media), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were seeded onto 24 mm (4.5 cm^2) transparent permeable membrane filters (Costar, Cambridge, MA, USA) and allowed to grow in the standard media for 7 days before experiment.

Measurement of electrical parameters

To investigate the long term effect of genistein on electrical parameters, the standard medium bathing the epithelial cell monolayers was changed to estrogen-free medium containing phenol red free DMEM, 5% charcoal-stripped FBS and other

supplements as in the standard medium (day 0). After 24 hr, genistein (1, 10 and 20 μM) or DMSO was added into the medium and allowed to grow for 4 days (day 1-4). The final concentration of DMSO in the solution was less than 0.01%. Transepithelial resistance of the cell monolayers was measured daily with an EVOM epithelial voltohmmeter coupled to Ag/AgCl 'chopstick' electrodes (World Precision Instruments, Serasota, FL). Following 4-day treatment, the monolayers with high resistance (≈ 3000 Ωcm^2) were then mounted in Ussing Chambers, bathed on both sides with standard porcine Ringer solution (in mM: 111.5 KCl, 25 $NaHCO_3$, 1.8 Na_2HPO_4, 0.2 NaH_2PO_4, 1.25 $CaCl_2$, 1.0 $MgSO_4$ and 12 glucose) which was maintained at 37°C and bubbled with 95%O_2 - 5%CO_2. Transepithelial potential difference (PD) and short circuit current (Isc) were measured with the use of voltage-clamp circuitry (EVC-1000, WPI) with Ag/AgCl electrodes connected to the bathing solution via agar bridges. Transepithelial conductance (G) was calculated using Ohm's law (G=Isc/PD). The monolayer was continuously short-circuited, except a brief interval of open-circuited readings for PD measurement before and after adding any chemicals. The data from the voltage clamp was connected to a MacLab 4S A/D converter and recorded with a 400 MHz PowerPC Macintosh. After mounting, the cell monolayer was equilibrated for at least 30 min to achieve a stable Isc before the addition of chemicals. Positive Isc corresponded to the movement of positive charge from the apical to basolateral compartments or movement of negative charge from the basolateral to apical compartments or a combination of both.

RT-PCR experiments

The PEG cells were subcultured onto cell culture dish. At 90% confluency, the standard medium was switched to the estrogen-free medium for 24 hrs. After being treated with genistein (1, 10, 20 μM) for 48 hrs, total RNA from the cells (~$4x10^6$ cell) was extracted using a Quantum Prep AquaPure RNA isolation kit (Bio-rad Laboratories Inc.,

USA). Using an iScript cDNA synthesis kit (Bio-rad Laboratories Inc., USA) to generate complimentary DNA (cDNA), 3 μg of total RNA was reversed transcribed in a 15 μl reaction volume according to the manufacturer's instruction. The reversed transcribed reaction was incubated at 25°C for 5 min, followed by 42°C for 30 min.

Relative quantitative real-time PCR across multiple samples were obtained using primer pairs of porcine SK1, SK2, SK3 (Palmer et al., 2008), CFTR and internal control GAPDH gene products as shown in Table 1. PCR reactions were prepared with 12.5 μl 2x SYBR® Green PCR Master Mix, 1 μl each of specific forward and reverse primers in a 25-μl reaction volume. RT reactions were performed with a StepOnePlus thermal cycler from Applied Biosystems (Foster City, CA, USA). Cycling conditions were performed as followed: 1 cycle to warm and hold at 95ºC for 10 min; 45 cycles of 95°C for 15 s, 60°C for 1 min, 72°C for 1 min; followed by a final end cycle at 72°C for 7 min. The dissociation curve (melting curve) was following performed to confirm specificity of amplified product using 95°C for 1 min and 59°C for 30 s. The specific PCR product for SK1, SK2, SK3, CFTR and GAPDH were considered when a single sharp peak was seen.

Table 1 Primer pairs of SK, CFTR and GAPDH

	Forward primer	Reverse primer
SK1	5'-GCA CATA CTT CAT GAT GGA CA-3'	5'-GTA CTT ACG CTG GTG TTA CC-3'
SK2	5'-CCC CGA GTT CGT GGT GTC TAA GC-3'	5'-CAC ACT CCA GTA TTT CCA AGC TGA TG-3'
SK3	5'-TCC ATG TTA TCG TTG GCC CTG-3'	5'-AGC ATG ACT CGG GCG ATC TGG TA-3'
CFTR	5' TCA TGC CGG GCA CCA TTA AA 3'	5' TTT GAT GAC ACT CCT GTA TCT A 3'
GAPDH	5'-TAC CTC GGC ACT GTC TA-3'	5'-GCT GCA CAG GTG CAC AA-3'

Data analyses

All values are presented as the mean ± standard error of mean (S.E.M.) and n is the number of monolayers used in each experiment. The increased I_{sc} in response to drug was the difference between peak I_{sc} response and its respective baseline value before drug administration. The statistical differences between control and treatment means were analyzed using a Student's t-test or Analysis of Variance (ANOVA) where appropriate. Difference between treatment and control means following a significant ANOVA was identified by Dunnett's test (Prism^{TM} 5.0, GraphPad Software, Inc., San Diego, CA). A *p-value* less than 0.05 was considered statistically significant.

RESULTS

Long-term effect of genistein on membrane permeability

Our previous studies have shown the direct acute effect of genistein on stimulating Cl^- secretion across the immortalized endometrial epithelial cells (PEG cells) (Deachapunya and Sutthasinee, 2010). To investigate the long term effect of genistein on ion transport, we first determined the transepithelial resistance (TER) across the PGE cell monolayer in the presence of genistein for 4 days. As shown in figure 1, cells growing in the estrogen-free medium showed a time dependent decrease in TER which was significantly observed on day 4 compared to day 0. Treatment with genistein 1 μM had no significant change from the vehicle control whereas genistein 10 μM or 20 μM prevented a reduction of TER on day 4 compared to the untreated group in the estrogen-free medium on day 0.

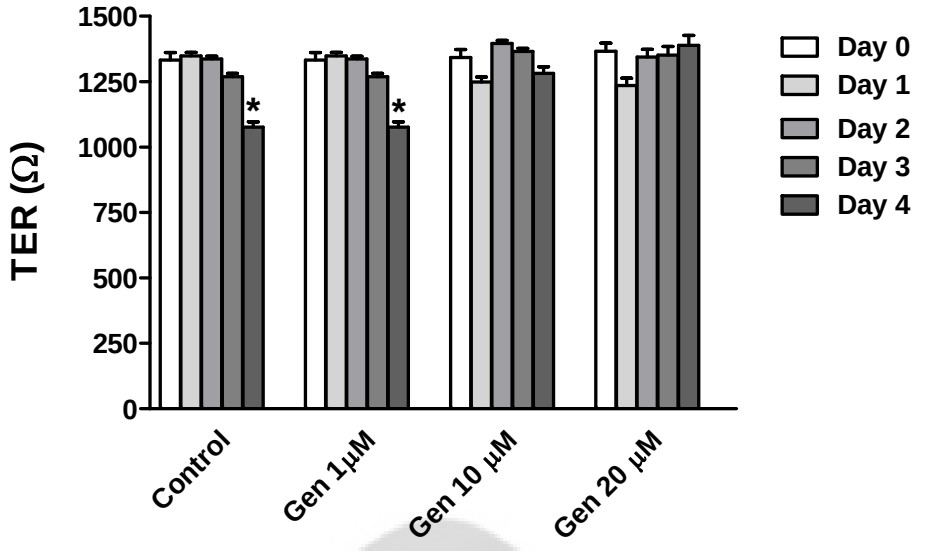

Figure 1 Changes in transepithelial resistance (TER) of the immortalized porcine endometrial epithelial cells. The cells were grown in the estrogen-free medium in the absence (Control) or presence of genistein (1, 10 and 20 μM) for 4 days. The data represented mean ± SEM (n = 12-16 monolayers). *P<0.05 when compared to the control experiments by student t's test.

Long-term effect of genistein on Cl^- secretion

When the PEG cells grown in the estrogen-free medium in the absence or presence of genistein for 4 days were mounted in Ussing chamber, averages of Isc, PD (lumen negative), conductance (G) and transepithelial resistance (R) were shown in table 2. Treatment with genistein (20 μM) produced no significant change in the baseline Isc and G while exhibiting a significant increase in the PD and R. To assess whether the long term treatment of genistein modulated Cl^- secretion, we stimulated Cl^- secretion with forskolin, UTP and prostaglandin E_2 in the genistein-treated monolayer. Forskolin, an activator of adenylate cyclase, stimulates Cl^- secretion via an increase in intracellular cAMP. In porcine endometrial epithelial cells, Cl^- secretion is stimulated by UTP via an increase in

intracellular Ca^{2+} (Palmer-Densmore et al., 2002) and by PGE_2 through CFTR Cl^- channels (Deachapunya and O'Grady, 1998). As illustrated in figure 2A, addition of forskolin (1 μM) to the apical and basolateral solution of the untreated monolayer produced a rapid increase in Isc followed by a gradual decrease to a sustained level above the baseline. The peak increased Isc induced by forskolin in cell monolayer treated with genistein 1 or 10 μM for 4 days did not differ from that in the untreated control monolayer (Fig 2B). However, monolayers treated with genistein 20 μM showed the peak Isc response to forskolin which was significantly less than those treated with genistein 1 μM or control group.

Table 2 Short circuit current (Isc), potential difference (PD), conductance and resistance of the immortalized endometrial epithelial cells. Cells were grown for 4 days in the estrogen-free medium in the absence (Control) or presence of genistein 1, 10 and 20 μM. The data represented mean ± SEM. *P<0.05 when compared to the control experiments by student t's test.

	n	Isc (μA)	PD (mV)	Conductance (mS)	Resistance (Ω)
Control	16	-2.99 ± 1.20	1.43 ± 0.73	2.09 ± 0.29	578.08 ± 52.91
Genistein 1 μM	21	-4.28 ± 1.30	2.95 ± 1.02	2.63 ± 0.49	602.80 ± 74.56
Genistein 10 μM	15	-4.89 ± 1.61	3.15 ± 1.43	2.77 ± 0.88	641.90 ± 88.41
Genistein 20 μM	13	-6.21 ± 1.97	5.64 ± 2.00*	1.53 ± 0.22	839.4 ± 113.61*

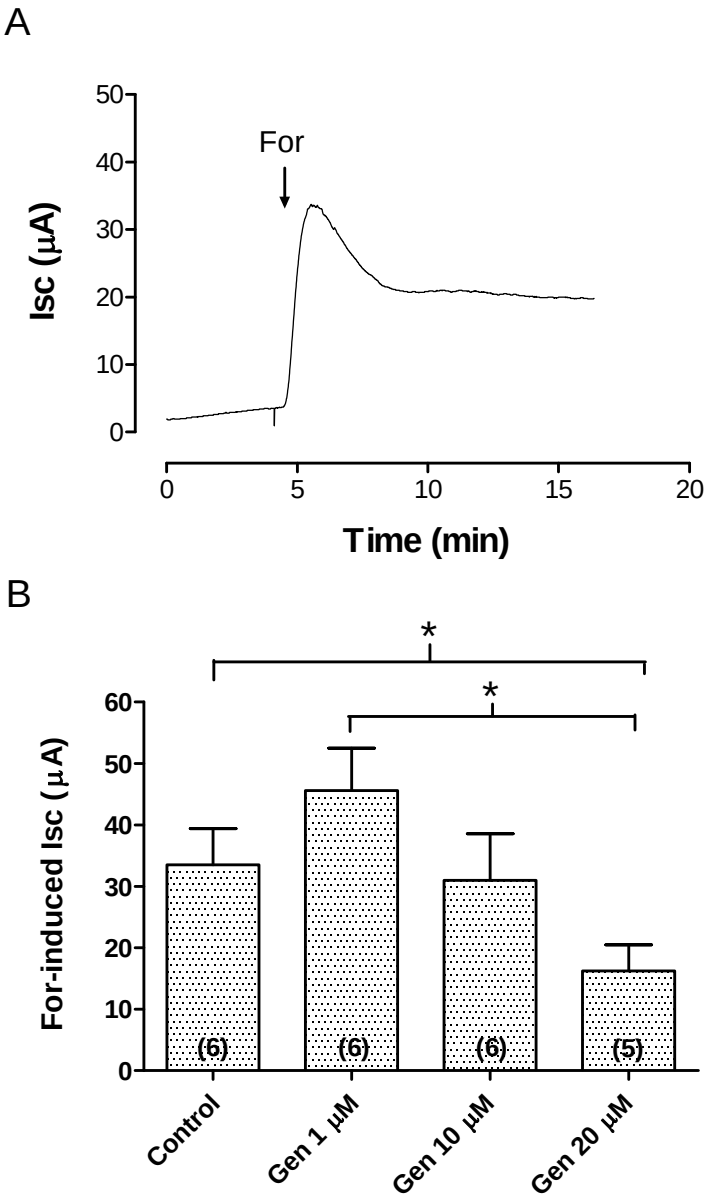

Figure 2 The effect of genistein on forskolin-activated Isc in the immortalized endometrial epithelial cells. A. Representative Isc tracings responded to forskolin (For 1 μM) was added to the apical and basolateral solution in cells grown in the estrogen-free medium. B. Bar graph showed the average change in the peak of Isc response to forskolin. The cells were grown for 4 days in the estrogen-free medium in the absence (Control) or presence of genistein (Gen) 1, 10 and 20 μM. Each value represented mean ± SEM. *P<0.01 when compared between two experiments by ANOVA. The number of monolayers is in parenthesis.

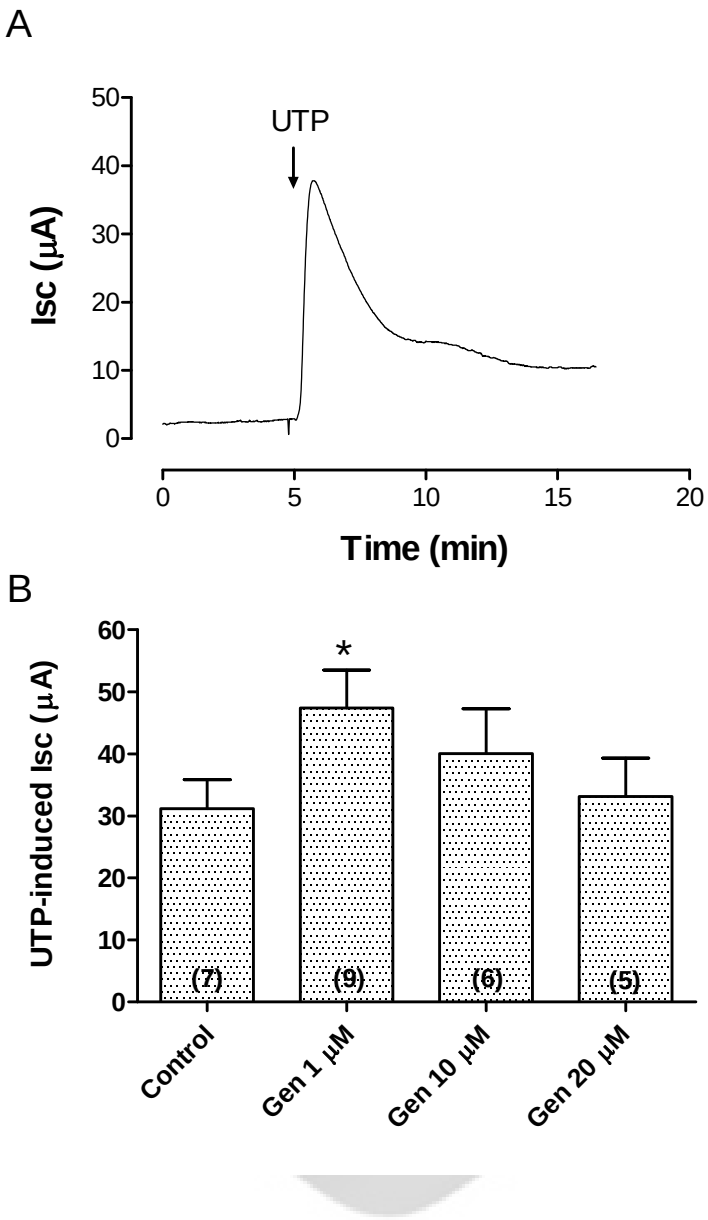

Figure 3 The effect of genistein on uridine triphosphate (UTP)-activated Isc in the immortalized endometrial epithelial cells. A. Representative Isc tracings responded to UTP (5 μM) was added to the apical solution in cells grown in the estrogen-free medium. B. Bar graph showed the average change in the peak of Isc response to UTP. The cells were grown for 4 days in the estrogen-free medium in the absence (Control) or presence of genistein (Gen) 1, 10 and 20 μM. Each value represented mean ± SEM. *P<0.05 when compared to the control by ANOVA. The number of monolayers is in parenthesis.

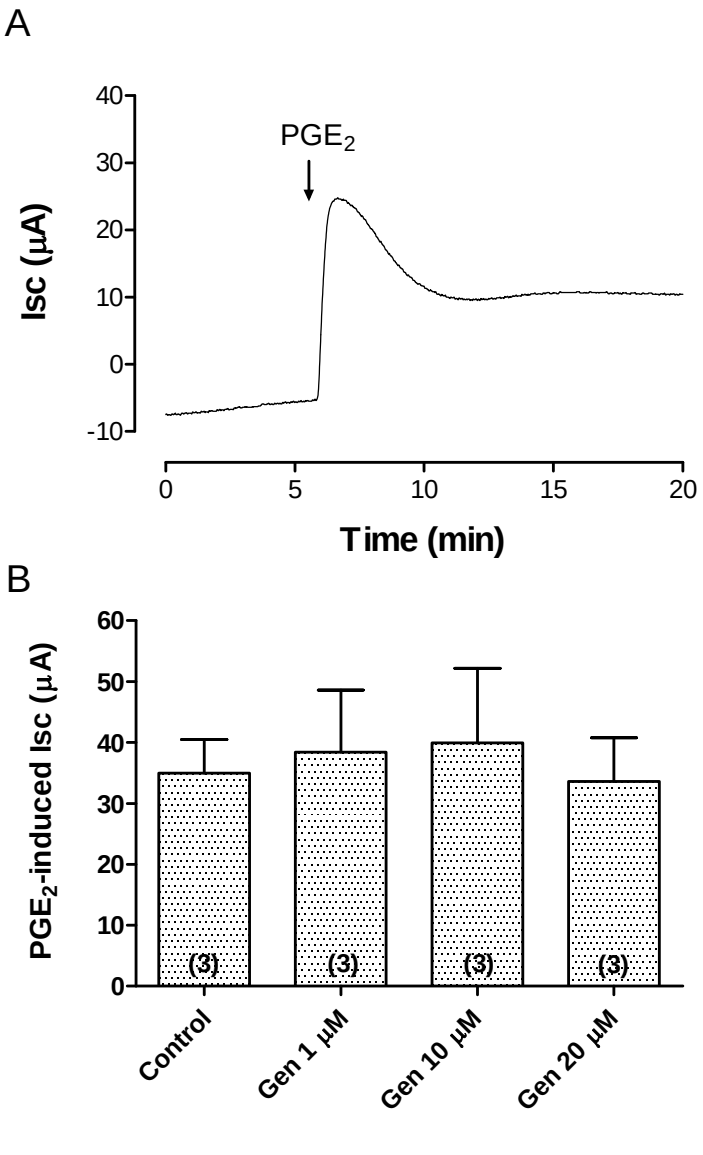

Figure 4 The effect of genistein on prostaglandin E_2 (PGE_2)-activated Isc in the immortalized endometrial epithelial cells. A. Representative Isc tracings responded to PGE_2 (3 μM) was added to the basolateral solution in cells grown in the estrogen-free medium. B. Bar graph showed the average change in the peak of Isc response to PGE_2. The cells were grown for 4 days in the estrogen-free medium in the absence (Control) or presence of genistein (Gen) 1, 10 and 20 μM. Each value represented mean ± SEM. The number of monolayers is in parenthesis.

UTP 1 μM when added to the apical solution also produced a transient increase in Isc and rapidly decreased almost to the baseline level as shown in figure 3A. Only genistein 1 μM showed a significant increase in the UTP-induced increase in Isc compared to the control (Fig 3B). Moreover, addition of prostaglandin E_2 to the basolateral solution produced an increase in Isc in a similar pattern as forskolin (Fig 4A). However, genistein treatment was without effect on the PGE_2-induced increase in Isc compared to the control as shown in figure 4B.

Long-term effect of genistein on the expression of Ca^{2+}-activated K^+ and CFTR mRNA

Our previous study using porcine endometrial epithelial cells have demonstrated that Cl^- secretion in response to genistein requires activation of both apical Cl^- and basolateral K^+ transport (Deachapunya and Sutthasinee, 2010). We therefore investigate the effect of genistein on the expression of small-conductance Ca^{2+}-activated K^+ channel (SK)1, SK2, SK3, and CFTR mRNA. The expression of SK mRNA has been identified in the PEG cells by Palmer and coworkers (2008). PCR products of appropriate size were detected for each primer set while none were observed in the absence of template or when RNA was substituted for cDNA (No RT). The fluorescent intensity of PCR products using SK1-3 primers demonstrated the expression of mRNA for the SK1, SK3 and less SK2 obtained from epithelial cells grown in the estrogen-free medium (Fig 5A). Analysis of mRNA expression as indicated by densitometry following normalization to corresponding GAPDH controls as illustrated in figure 5B-D revealed that SK1 and SK2 mRNA expression were significantly increased by genistein treatment at 10 μM for 2 days. However, high concentration of genistein (10 μM) decreased SK3 mRNA expression compared to the vehicle control. On the other hand, the PCR products of CFTR primers obtained from epithelial cells grown in the estrogen-free medium or in the presence of genistein was illustrated in figure 6A. The normalized CFTR mRNA intensity compared

to corresponding GAPDH mRNA was increased in cells treated with genistein 10 μM while a reduction in the CFTR expression was observed by genistein 20 μM compared to the control (Fig 6B).

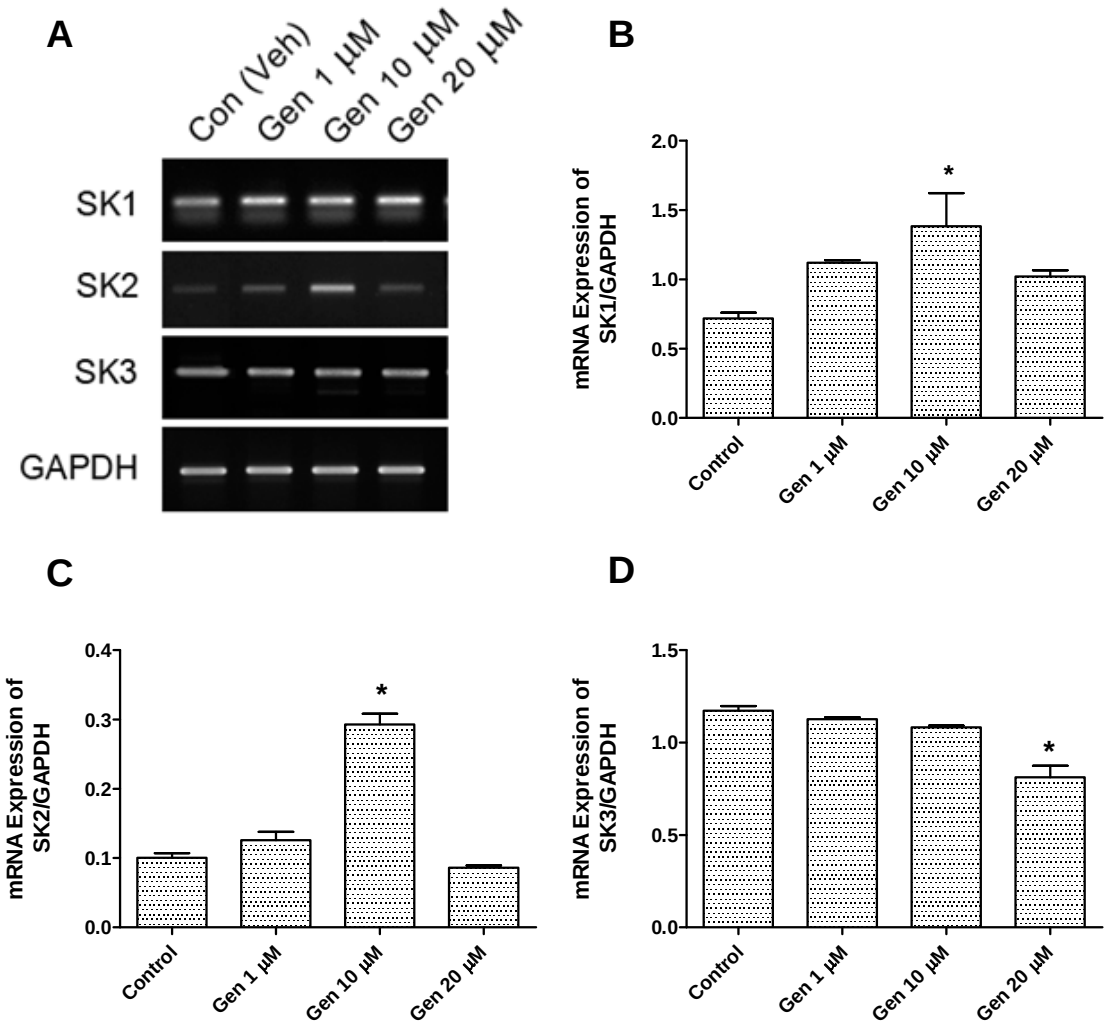

Figure 5 Quantitative RT-PCR analysis of SK1, SK2 and SK3 mRNA expression in the immortalized endometrial epithelial cells. Cell monolayers were maintained for 2 days under phenol red-free medium supplemented with charcoal-stripped FBS in the presence of genistein (Gen 1, 10, 20 μM) or vehicle control. Values are mean ± SEM of 3 monolayers. *P<0.05 when compared among groups by ANOVA.

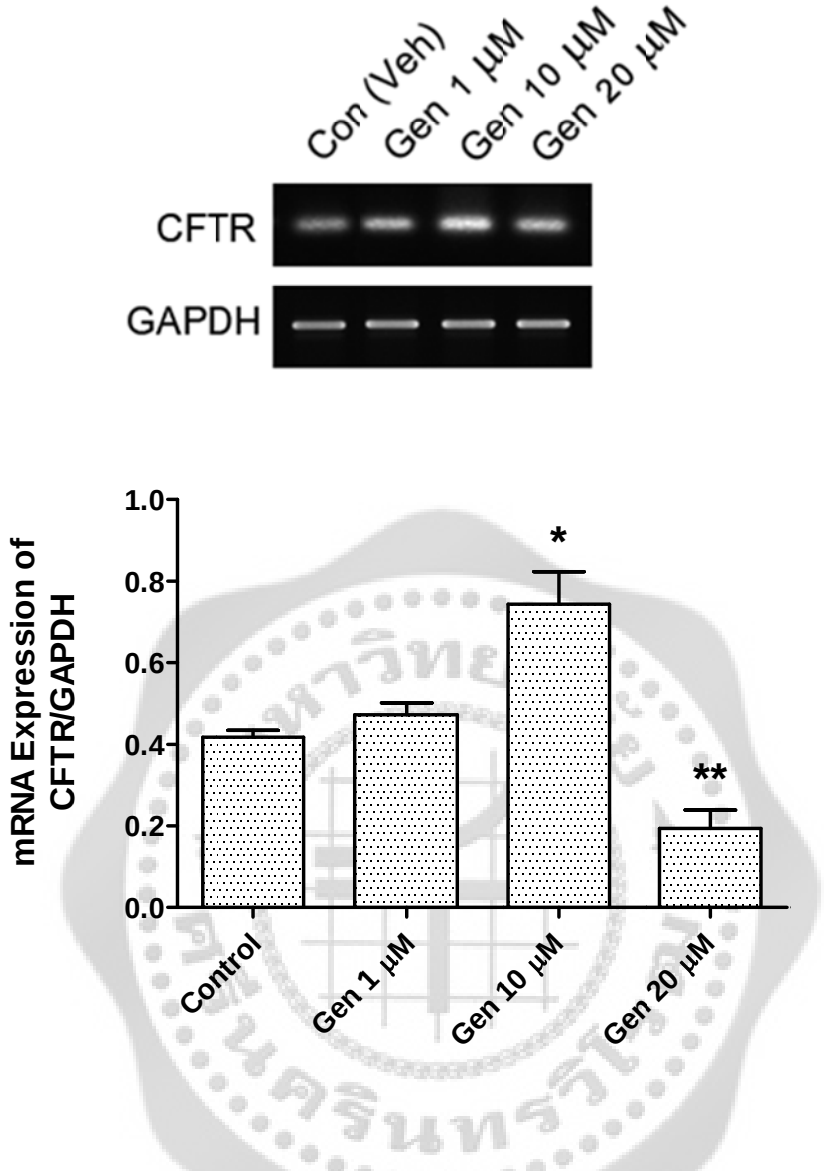

Figure 6 Quantitative RT-PCR analysis of CFTR mRNA expression in the immortalized endometrial epithelial cells. Cell monolayers were maintained for 2 days under phenol red-free medium supplemented with charcoal-stripped in the presence of genistein (Gen 1, 10, 20 μM) or vehicle control for 4 days. Values are mean ± SEM of 3 monolayers. *P<0.05, **P<0.01 when compared among groups by ANOVA..

DISCUSSION

Our previous studies using immortalized porcine endometrial epithelial cells demonstrated the acute effect of genistein on activating Cl^- secretion (Deachapunya and Poonyachoti, 2010). In the present study, treatment of genistein at high concentrations was found to maintain membrane integrity of the endometrial epithelium. Moreover, genistein appeared to modulate the Cl^- secretion mediated by intracellular cAMP and Ca^{2+} as well as the gene expression of small conductance Ca^{2+}-activated K^+ and cAMP-activated Cl^- channels.

In epithelia, the transepithelial resistance (TER) and integrity of the tissue are determined by the formation and permeability of tight junctions and potential difference (PD) as determined by epithelial cell transport. A decrease in TER and PD reflects decreased membrane integrity and transport functions. In the present study, monolayers cultured in estrogen-free medium which consisted of phenol red-free medium supplemented with charcoal-stripped serum for 4 days were associated with a marked reduction in TER observed on day 4, suggesting that estrogen is required for maintaining membrane integrity. However, addition of genistein especially at concentrations of 10-20 μM in the estrogen-free medium prevented a reduction in membrane integrity. These findings were correlated with the electrophysiological parameters as determined by Ussing chamber experiment following 4-day treatment which revealed that genistein at high concentration significantly increased the basal PD and resistance (Table 2). These findings indicated that genistein can be used as estrogen supplement to maintain the membrane integrity across the endometrial epithelium.

In the present study, as the basal PD and resistance being increased, however, the basal Isc and conductance were not affected by high concentration of genistein. This was opposite to stimulation the basal Isc as observed in the study with the acute genistein

effect. Nevertheless, exposure to genistein 20 μM for 4 days was found to decrease Cl^- secretion through CFTR which was stimulated by forskolin via an increase in cAMP. The reduction of the forskolin-induced increase in Isc was associated with suppression of CFTR mRNA expression. In the acute study, genistein produced a biphasic response as shown by changes in the Isc and the apical Cl^- current. At concentrations of genistein lower than 50 μM, both Isc and apical Cl^- current were stimulated in a concentration dependent manner, but were inhibited at high concentration of genistein (100 μM). An inhibitory effect of genistein are also observed in rat distal colon and nasal epithelial tissues (Deiner and Hug, 1996; Malls et al., 2000), which is possibly due to inhibition of K^+ channels. In our previous study, genistein when applied at higher concentrations was shown to inhibit both apical K^+ current and apical Cl^- current, suggesting an inhibitory effect of genistein was contributed by inhibitions of both Cl^- and K^+ channels (Deachapunya and Poonyachoti, 2009). Therefore, in the present study, a reduction in the forskolin-induced increase in Cl^- secretion in the genistein-treated monolayers was likely due to the suppression of CFTR by high concentration of genistein.

In the porcine endometrial epithelial cells, UTP has been demonstrated to stimulate Cl^- secretion via an increase in intracellular Ca^{2+} and PKC stimulation (Palmer-Densmore et al., 2002). Recent study has demonstrated that UTP-induced increase in Isc was completely abolished in the presence of genistein. This could be explained by an inhibitory effect of genistein on the apical K^+ current and basolateral K^+ current that are activated by UTP (Deachapunya and Poonyachoti, 2009). The previous study with the acute effect of genistein was inconsistent with the present study in which chronic treatment of genistein was found to stimulate Cl^- secretion induced by UTP. These findings indicate that long term treatment of genistein may modulate the transport mechanism or transporter responsible for UTP-stimulated Cl^- secretion.

Since a number of transporter proteins involving Cl^- secretion including the cystic fibrosis transmembrane conductance regulator (CFTR), K^+ channels, and Na^+-K^+-$2Cl^-$ cotransporter have been modulated by genistein (Diener and Hug, 1996; Illek et al., 1996; Niisato et al., 1999), we therefore primarily investigated the long term effect of genistein on the expression of small-conductance Ca^{2+}-activated K^+ channel (SK) and CFTR mRNA in the porcine endometrial epithelial cells. The SK mRNA is expressed in the PEG cells and shown to be modulated by estrogen treatment (Palmer et al., 2002). Our findings revealed that treatment of genistein increased SK1 and SK2, but decreased SK3 mRNA, suggesting the genomic effects of genistein on modulation of the Ca^{2+}-activated K^+ channels in the endometrial epithelial cells. Although the increases in SK1 and SK2 expression were markedly increased by genistein 10 μM, this seemed to be correlated with the increased Cl^- secretion induced by UTP in 1 μM genistein-treated monolayer. Generally, the increase in K^+ efflux through K^+ channels is known to hyperpolarize the membranes, thus providing the driving force for Cl^- secretion. Moreover, the rise in SK current also enhances Ca^{2+} entry to stimulate the intracellular mechanisms that account for cell proliferation (Yamazaki et al., 2006). Therefore, we speculate that the increase in SK1 and SK2 expression in response to long term regulation of genistein would be a mechanism for increasing the driving force for Cl^- secretion following UTP stimulation. Nevertheless, a question still remained about the suppressive effect of genistein on SK3 expression on Cl^- secretion.

Other and our previous studies have been proposed the action of genistein on a direct activation of CFTR (French et al., 1997; Weinreich et al., 1997; Deachapunya and Poonyachoti, 2010). In the present study, the 4-day treatment of genistein showed a biphasic effect on CFTR mRNA expression, i.e. 1 and 10 μM increased the CFTR mRNA expression whereas 20 μM genistein decreased expression. The suppressed CFTR mRNA

expression was well associated with a reduction of Cl^- secretion induced by forskolin as previously mentioned. By contrast, estrogen treatment has been reported to upregulate the CFTR expression (Rochwerger et al., 1994). This could be due to the different signaling mechanism of estrogen and phytoestrogen genistein on the regulation of CFTR gene expression. In the present study, we speculate that the decrease in CFTR mRNA in response to high concentration of genistein would be responsible for an inhibition of Cl^- secretion following forskolin stimulation. Nevertheless, the genomic effects of genistein regulation of other transport-related genes including Ca^{2+}-activated Cl^- channels, cAMP-activated K^+ channels and Na^+-K^+-$2Cl^-$ cotransporter in the endometrial epithelial cells are required for further studies.

In conclusion, the results from the present study indicated that the isoflavone genistein could be used as supplement to maintain the membrane integrity across the endometrial epithelial cells. Although genistein had no significant role on the basal ion transport, it appeared to modulate the Cl^- secretion mediated by intracellular cAMP and Ca^{2+}. The genomic action of genistein on Cl^- secretion was associated with upregulation of SK1 and SK2 mRNA expression and suppression of CFTR mRNA expression which were responsible for activation of Ca^{2+}-activated Cl^- secretion and inhibition of cAMP-activated Cl^- secretion, respectively. Taken together these results may be of benefit in the use of isoflavone genistein as a pharmacotherapeutic agent for long term modulation of electrolyte transport in the endometrial epithelium.

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