Original Paper

Activation of Chloride Secretion by Isoflavone Genistein in Endometrial Epithelial Cells

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Key Words

Daidzein • Phytoestrogen • Chloride transport • UTP • Endometrial epithelium

Abstract

Background /Aim: Genistein, the most active isoflavone found primarily in soybeans, alters ion transport functions in intestinal and airway epithelia. The present study aims to investigate the acute effects and mechanisms of action of genistein in immortalized porcine endometrial epithelial cells. **Methods:** Ussing chamber technique was used for transepithelial electrical measurements. **Results:** Genistein increased short-circuit currents (I_{sc}) which were inhibited by glibenclamide, NPPB, CFTRinh-172, DIDS or bumetanide, but not amiloride. In experiments with amphotericin B-permeabilized monolayers, genistein activated the apical Cl^- current and barium-sensitive basolateral K^+ current while inhibiting the apical K^+ current. Genistein failed to increase the I_{sc} in the presence of forskolin or IBMX, but did increase the I_{sc} in UTP. Pretreatment with genistein also abolished the increase in the I_{sc} when induced by forskolin, IBMX or UTP. However, Ca^{2+} -chelating BAPTA-AM did not affect the genistein-induced increase in the I_{sc} . The genistein-stimulated I_{sc} was reduced by tyrosine kinase inhibitors, tyrphostin A23 or AG490. However, vanadate, a tyrosine phosphatase inhibitor, failed to inhibit the genistein response. Estrogen receptor antagonist ICI182,780 did not alter the genistein's action. **Conclusion:** The soy isoflavone, genistein, stimulates Cl^- secretion in endometrial epithelial cells possibly via a direct activation of CFTR which appears to be modulated through a tyrosine kinase-dependent pathway. The present findings may be of benefit for the therapeutic application of genistein in the treatment of electrolyte transport disorders in the epithelia.

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Introduction

Genistein is a naturally occurring isoflavone found primarily in leguminous plants with soybeans and its by products with especially high concentrations [1]. As its chemical structure relates to estradiol, an endogenous estrogen, genistein can bind to estrogen receptors [2] and exert weak estrogenic and/or antiestrogenic effects on numerous cells and tissues both *in vitro* and *in vivo* [3]. Consuming soy-based foods has been suggested to have potential health benefits; for example, reduced risks of cardiovascular diseases, lower blood pressure, reduced incidences of osteoporosis, and reduced risks for breast and endometrial cancers [4, 5]. However, its underlying mechanisms are unclear and some adverse effects of genistein on the female reproductive tract have been reported [6, 7].

The regulation of electrolyte composition and fluid volume within the uterine cavity provides an optimal intrauterine environment that is required for the reproductive events of gamete transport and embryo development [8]. Sex-steroid hormones, estrogen and progesterone, are associated with local factors of cytokines and growth factors that orchestrate the control of the activities of the epithelia's surface and glandular transport systems [8, 9]. Alteration in uterine ionic composition observed in hydrosalpinx or cystic fibrosis have been associated with many reproductive failures, pathologic reproductive conditions, infertility and recurrent pregnancy losses [10].

The isoflavone genistein has been shown to alter ion transport in both native epithelia and cultured epithelial cells. It stimulates Cl⁻ secretion in rat colons, mouse intestines, human colonic cell lines and airway epithelia [11-14]. A number of transporter proteins involved in Cl⁻ secretion including the cystic fibrosis transmembrane conductance regulator (CFTR), K⁺ channels, and Na⁺-K⁺-2Cl⁻ cotransporters have been modulated by genistein [11, 12, 15]. Recently, genistein has been proposed as a promising therapeutic agent for cystic fibrosis through its modulation of transport proteins involved in Cl⁻ secretion [16].

Several molecular mechanisms of genistein's action on CFTR activity have been proposed. These include the modulation of tyrosine kinases [17], protein phosphatases [18], protein kinases [19] and the direct activation of CFTR [17, 20]. Genistein has been shown to activate the CFTR via a tyrosine-dependent phosphorylation pathway as its effect is abolished by the tyrosine phosphatase inhibitor vanadate [11, 14, 21]. However, genistein also stimulates the CFTR in the presence of vanadate [22]. In addition, tyrphostin A23 and tyrphostin A51, tyrosine kinase inhibitors that are structurally different from genistein, do not activate Cl⁻ secretion in T84 cells [23]. In addition, a tyrphostin A23 pretreatment is found to reduce the genistein-stimulated Cl⁻ secretions in rat jejunums [24]. Other studies have demonstrated a direct interaction of genistein with CFTR at nuclear binding domain 2 via tyrosine kinase- and protein phosphatase independent mechanisms [17, 20]. In CFTR overexpressing cells, genistein has been shown to produce a biphasic effect on CFTR gating (i.e., stimulatory effects at low concentrations and inhibitory effects at high genistein concentrations [22]). Thus, the role of genistein on the phosphorylation and dephosphorylation-dependent regulation of CFTR is not well defined and appears to be tissue or cell-specific and concentration dependent. Other mechanisms of genistein's action involve an increased CFTR localization to the plasma membrane [25, 26] and a stimulation of Cl⁻ secretion via inhibition of the phosphodiesterase-dependent pathway [24].

Electrophysiological studies of both intact endometrial epithelium and cultured endometrial epithelial cells have provided direct evidence for the regulation of Na⁺ absorption, K⁺ secretion [27-30] and Cl⁻ secretion [31-33]. Although genistein exerts transport-related effects on a variety of epithelial cells, little is known regarding the direct effects of genistein on the ion transport functions of the endometrial epithelium. Therefore the aim of this study is to investigate the acute effects and the mechanism of action of genistein on ion transport function in an immortalized porcine endometrial epithelial cell line. These cells express estrogen receptors both alpha and beta subtype (unpublished data) and exhibit functionally characterized CFTR and other transport proteins for Cl⁻ and K⁺ secretion [34, 35]. This

provides a suitable model for studying the transport-related properties of estrogen-like substances.

Materials and Methods

Materials

Genistein, daidzein, forskolin, 3-isobutyl-1-methylxanthine (IBMX), uridine-5'-triphosphate (UTP), amiloride, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), glibenclamide, 4,4'-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS), bumetanide, 1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid (BAPTA-AM), tyrphostin A23, AG490, sodium orthovanadate (vanadate), 7 α -[9-(4,4,5,5,5-pentafluoro-pentylsulphonyl)nonyl]oestra-1,3,5(10)-triene-3,17 β -diol (ICI182,780), 17 β -estradiol, BaCl₂, 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), fetal bovine serum (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 gland 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 [35]. The PEG cells (passage number 62-80) were cultured in DMEM supplemented with 3.7 g/L NaHCO₃, 10% heat-inactivated FBS, 850 nM (5 μ g/ml), insulin, 1% non-essential amino acid, 100 U/ml penicillin, 100 μ g/ml streptomycin and 100 μ g/ml kanamycin, and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells were then seeded onto 24 mm (4.5 cm²) transparent permeable membrane filters (Costar, Cambridge, MA, USA) for 7-10 days prior to the experiment.

Measurement of electrical parameters

Transepithelial resistance of cell monolayers was measured with an EVOM epithelial volttohmmeter coupled to Ag/AgCl 'chopstick' electrodes (World Precision Instruments, Sarasota, FL, USA). Monolayers with high resistance ($\approx$ 3000 Ω cm²) were then mounted in Ussing Chambers, bathed on both sides with standard porcine Ringer solution (in mM: 111.5 KCl, 25 NaHCO₃, 1.8 Na₂HPO₄, 0.2 NaH₂PO₄, 1.25 CaCl₂, 1.0 MgSO₄ and 12 glucose) which was maintained at 37°C and bubbled with 95%O₂ - 5%CO₂. Transepithelial potential difference (PD) and short circuit current (I_{sc}) were measured with a voltage-clamp circuitry (EVC-4000, WPI) with Ag/AgCl electrodes connected to the bathing solution via agar bridges. Transepithelial conductance (G) was calculated using the Ohm's law ($G = I_{sc} / PD$). The monolayer was continuously short-circuited, except for a brief interval of open-circuited readings for PD measurements before and after the addition of any chemicals. Data from the voltage clamp was connected to a MacLab 4S A/D converter and then the data was stored in a Macintosh 400 MHz PowerPC. After mounting on Ussing chamber, the monolayer was equilibrated for a minimum of 30 minutes to achieve a stable I_{sc} before the addition of test substances. A positive I_{sc} corresponded with the movement of the positive charge from the apical to the basolateral compartments or resulted from the movement of negative charges from the basolateral to apical compartments or were the result of a combination of both processes.

In the experiments with measurement of membrane permeability, an apical membrane current was determined by permeabilization of the basolateral membrane of the monolayers mounted in the Ussing chambers with amphotericin B (10 μ M). The permeabilized basolateral membrane was bathed with a KMeSO₄ Ringer solution (in mM: 120 KMeSO₄, 30 mannitol, 5 NaCl, 3 calcium gluconate, 1 MgSO₄, 20 KHCO₃, 0.3 KH₂PO₄, 1.3 K₂HPO₄) while the apical membrane was bathed with a high concentrate KCl Ringer solution (in mM: 120 KCl, 30 mannitol, 5 NaCl, 3 calcium gluconate, 1 MgSO₄, 20 KHCO₃, 0.3 KH₂PO₄, 1.3 K₂HPO₄) for apical Cl⁻ current (I_{Cl}) measurements or with NaMeSO₄ Ringers solution (in mM: 120 NaMeSO₄, 30 mannitol, 5 NaCl, 3 calcium gluconate, 1 MgSO₄, 20 KHCO₃, 0.3 KH₂PO₄, 1.3 K₂HPO₄) for apical K⁺ current (I_{Ka}) measurements. The basolateral membrane K⁺ current (I_{Kb}) measurement was performed by amphotericin B-permeabilized apical membrane bathed with a KMeSO₄ Ringer solution, and the basolateral membrane was bathed with a NaMeSO₄ Ringer solution. All solutions were adjusted to a pH of 7.4 at a temperature of 37°C.

Fig. 1. Effects of genistein and daidzein on basal I_{sc} of the immortalized porcine endometrial epithelial cells. Representative I_{sc} tracings in response to (A) genistein (100 μ M, apical and basolateral), (B) daidzein (50 and 100 μ M, apical and basolateral) and subsequently to genistein. (C) Changes in I_{sc} induced by genistein at concentrations of 0.1–100 μ M. (D) Analysis of concentration-dependent responses for the genistein-induced increase in I_{sc} show the EC_{50} value of 9.39 μ M ($n=5$).

Fig. 2. Effects of Na^+ and Cl^- channel blockers and $Na^+-K^+-2Cl^-$ cotransport blockers on the genistein-stimulated I_{sc} of the immortalized endometrial epithelial cells. Bar graph shows the average changes in I_{sc} as induced by genistein (Gen; 50 μ M, apical and basolateral) alone and in the presence of the Na^+ channel blocker amiloride (Amil; 10 μ M, apical), Cl^- channel blockers NPPB (100 μ M, apical), glibenclamide (Gli; 200 μ M, apical), CFTRinh-172 (20 μ M, apical) or DIDS (200 μ M, apical), or $Na^+-K^+-2Cl^-$ cotransporter blocker bumetanide (Bum; 200 μ M, basolateral). Each value represents mean $\pm$ SEM ($n=3-6$). * $P<0.01$ when compared to the control group with genistein alone by Dunnett's test.

Data analyses

Data was presented as a mean $\pm$ standard error of mean (S.E.M.), and n was the number of monolayers used in each experiment. The statistical differences between the control and experimental means were analyzed using a Student's t -test or Analysis of Variance (ANOVA) when appropriate. The difference between the treatment and the control mean, following a significant ANOVA, was identified by the Dunnett's test (Prism™ 5.00, GraphPad Software, Inc., San Diego, CA). A value of $P<0.05$ was considered statistically significant.

Results

Genistein-induced increase in I_{sc}

Under basal conditions, PEG cell monolayers showed an average I_{sc} , potential difference (lumen negative) and transepithelial conductance values of -0.62 ± 0.24 μ A/cm², 2.83 ± 0.89 mV and 0.22 ± 0.04 mS/cm² ($n=40$), respectively. The addition of genistein (100 μ M) to the apical and basolateral solutions produced a transient increase in the I_{sc} with a

Fig. 3. Effect of genistein on the cAMP-activated Cl⁻ secretion of the immortalized endometrial epithelial cells. (A-B) Representative I_{sc} tracings in response to genistein (Gen; 50 μ M, apical and basolateral) in the presence of forskolin (For; 10 μ M, apical and basolateral) or IBMX (100 μ M, apical and basolateral). (C) Average changes in the I_{sc} in response to genistein alone and in the presence of forskolin or IBMX. (D-E) Representative I_{sc} tracings responding to forskolin (10 μ M) or IBMX (100 μ M) in the presence of genistein (50 μ M). (F) Average changes in I_{sc} in response to forskolin or IBMX alone and in the presence of genistein. Each value represents mean $\pm$ SEM (n=4-6). *P<0.01 when compared to the corresponding control groups with compounds alone by Dunnett's test.

maximum effect of $7.41 \pm 0.87 \mu$ A/cm² within 2-3 min, followed by a gradual decrease in the I_{sc} and was sustained at $2.17 \pm 0.79 \mu$ A/cm² above the baseline (n=9, Fig. 1A). By contrast, the addition of daidzein (50 and 100 μ M), an inactive analog of genistein, had shown a relatively small stimulatory effect on the basal I_{sc} . A subsequent addition of genistein (100 μ M) produced a further increase in the I_{sc} (Fig. 1B). The accumulative I_{sc} response induced by genistein was concentration dependent with an EC_{50} value of 9.39 μ M and a maximum response at a concentration of 50 μ M (Fig. 1C-D). As genistein at a higher concentration (100 μ M) did not increase the I_{sc} further, the maximum response concentration of genistein (50 μ M) was used in the rest of the experiments. No changes in I_{sc} were observed when the DMSO vehicle control was added at any point in time.

Genistein-stimulated Cl⁻ secretion

Previous studies have demonstrated the effects of genistein on modulating Cl⁻ secretion in a variety of epithelia. Therefore, a series of experiments using various pharmacological channel blockers was conducted to determine the ionic basis involved in the increased I_{sc} in response to genistein. As shown in Fig. 2, the increased I_{sc} in response to genistein (50 μ M) was not affected by the presence of the apical Na⁺ channel blocker 10 μ M amiloride. By contrast, the genistein response was mostly abolished in the presence of 100 μ M NPPB or 200 μ M glibenclamide, well-known cAMP-activated CFTR blockers, or 20 μ M CFTRinh-172, a specific CFTR blocker, in the apical solution. The presence of 200 μ M DIDS, a Ca²⁺-activated

Fig. 4. Effect of genistein on the UTP-activated Cl⁻ secretion of the immortalized endometrial epithelial cells. (A) Representative I_{sc} tracing in response to genistein (50 μM, apical and basolateral) in the presence of UTP (5 μM, apical), and (B) Average changes in the I_{sc} responding to genistein alone and in the presence of UTP, (C) Representative I_{sc} tracing in response to UTP in the presence of genistein. (D) Average changes in the I_{sc} responding to UTP alone and in the presence of genistein. Each value represents mean ± SEM (n=4-6). *P<0.01 when compared to the control group with UTP alone by Student t-test.

Cl⁻ channel blocker also reduced the genistein-stimulated I_{sc} by 65%. In addition, blocking Cl⁻ loading across the basolateral membrane with 200 μM bumetanide, an inhibitor of Na⁺-K⁺-2Cl⁻ cotransport, completely inhibited the increase in I_{sc} induced by genistein (Fig. 2).

To assess the involvement of genistein in the cAMP-activated Cl⁻ secretion, we examined the effect of genistein in the presence of forskolin or IBMX, activators of CFTR via cAMP. As shown in Fig. 3, addition of 10 μM forskolin or 100 μM IBMX to both the apical and basolateral solutions markedly increased the I_{sc}. The subsequent addition of 50 μM genistein in the presence of forskolin or IBMX produced a slight further increase in the I_{sc} as compared to the results of the addition of genistein alone (Fig. 3A-C). On the other hand, pretreatment with genistein also abolished the increased I_{sc} induced by forskolin or IBMX (Fig. 3D-F).

Cl⁻ secretion across the porcine endometrial epithelial cells has been stimulated by UTP via an increase in intracellular Ca²⁺ [33]. To further investigate the effect of genistein on the Ca²⁺-activated Cl⁻ secretion, UTP was applied in the absence and in the presence of genistein. As shown in Fig. 4A, an apical addition of 5 μM UTP caused a rapid increase in the I_{sc} with a peak of 11.72 ± 2.23 μA/cm² followed by a sustained decrease in the I_{sc} at 1.27 ± 0.38 μA/cm² (n=4) above the baseline level. A subsequent addition of genistein produced a further increase in the I_{sc} which was not significantly different from the value observed when giving genistein alone (Fig. 4B-C). In contrast, the peak I_{sc} response induced by UTP was completely abolished after pretreatment with genistein (Fig. 4C-D).

Effects of genistein on membrane permeability

To assess the direct effects of genistein on apical and basolateral membrane permeability, we used amphotericin B (10 μM) to permeabilize one membrane of the cell

Fig. 5. Effect of genistein on membrane permeability of the immortalized endometrial epithelial cells. Representative tracings show the effects of genistein at concentrations of 10, 50 and 100 μM on (A) the apical membrane Cl^- current (I_{Cl}), (B) the apical membrane K^+ current (I_{K_a}), and (C) the basolateral membrane K^+ current (I_{K_b}). Experiments were performed in an amphotericin B-permeabilized monolayer bathed in Cl^- or K^+ gradient solutions (see methods).

Fig. 6. Effect of Ca^{2+} chelating BAPTA-AM, tyrosine kinase inhibitors, tyrosine phosphatase inhibitor, and selective estrogen receptor inhibitor on the genistein-stimulated I_{sc} of the immortalized endometrial epithelial cells. Bar graph shows the average changes in the I_{sc} in response to genistein (Gen; 50 μM) alone and in the presence of (A) BAPTA-AM (50 μM), (B) tyrphostin A23 (Tyr 23; 100 μM), AG490 (50 μM) or vanadate (100 μM), and (C) ICI182,780 (1 μM). All compounds were added to both, apical and basolateral solutions. Each value represents mean $\pm$ SEM ($n=3-6$). * $P<0.01$ when compared to the control group with genistein alone by Dunnett's test.

monolayers in order to exclusively study the permeability of the other membrane. As shown in Fig. 5A, when the basolateral membrane was permeabilized and bathed in a Cl^- -gradient solution, genistein (10 and 50 μM) caused concentration-dependent increases in the apical Cl^- current (I_{Cl}) as represented by the negative deflection of the current. Genistein at 50 μM produced a maximum I_{Cl} by $-8.69 \pm 1.39 \mu A/cm^2$ ($n=8$) whereas high concentration of genistein (100 μM) rapidly and slightly decreased the I_{Cl} . In a K^+ -gradient solution, genistein produced a concentration-dependent inhibition of the apical K^+ current (I_{K_a}) as was shown by an increase in the current (Fig. 5B). Genistein at 50 μM slightly inhibited I_{K_a} by 2.18 ± 0.61

$\mu\text{A}/\text{cm}^2$ ($n=7$). When the apical membrane was permeabilized with amphotericin B, low concentrations of genistein ($10 \mu\text{M}$) slightly increased the basolateral K^+ current (I_{Kb}) while high concentrations (50 and $100 \mu\text{M}$) elicited an immediate increase in the I_{Kb} followed by a gradual decrease in the I_{Kb} which could not retain a steady state at a concentration of $100 \mu\text{M}$ (Fig. 5C). Genistein at $50 \mu\text{M}$ increased the I_{Kb} by $2.90 \pm 0.49 \mu\text{A}/\text{cm}^2$ within 2-3 min and then gradually decreased to a sustained level below the baseline at $-17.06 \pm 4.12 \mu\text{A}/\text{cm}^2$ ($n=6$). Basolateral pretreatment with 5 mM Ba^+ , a non-specific K^+ channel blocker, abolished the increased I_{Kb} and inhibited the sustained level response to genistein to $-8.76 \pm 2.69 \mu\text{A}/\text{cm}^2$ ($n=3$).

Mechanism of genistein action on Cl^- secretion

To assess whether genistein activated Cl^- secretion was mediated by intracellular Ca^{2+} , the genistein response was tested in the presence of BAPTA-AM, a cell permeable intracellular Ca^{2+} chelating agent. Pretreatment of the monolayer with $50 \mu\text{M}$ BAPTA-AM in the apical and basolateral solutions for 30 min slightly decreased the baseline and did not affect the genistein-induced increase in the I_{sc} (Fig. 6A). However, BAPTA-AM completely inhibited the increase in the I_{sc} induced by UTP ($-0.25 \pm 0.05 \mu\text{A}/\text{cm}^2$, $n=3$). These results suggest that the genistein response does not involve Ca^{2+} mobilization.

Since Cl^- secretion through CFTR has been shown to be regulated by phosphorylation and dephosphorylation of the nucleotide binding domain, we further examined the effect of tyrosine kinase inhibitors, tyrphostin A23 or AG490, or tyrosine phosphatase inhibitor, vanadate, on the genistein-stimulated I_{sc} response. The experiment was performed by the pretreatment of the monolayers with inhibitors added to the apical and basolateral solutions for 30 min prior to the genistein application. Tyrphostin A23 ($100 \mu\text{M}$) decreased the baseline I_{sc} by $-0.76 \pm 0.19 \mu\text{A}/\text{cm}^2$ ($n=4$) and significantly reduced the genistein-induced increase in the I_{sc} by 80% (Fig. 6B). Like tyrphostin A23, AG490 ($50 \mu\text{M}$) had no effect on the basal I_{sc} and markedly abolished the genistein-increased I_{sc} by 93% ($n=4$). Addition of vanadate ($100 \mu\text{M}$) slightly increased the baseline I_{sc} by $0.66 \pm 0.30 \mu\text{A}/\text{cm}^2$ ($n=4$); however, it failed to inhibit the genistein-induced increase in the I_{sc} .

To further investigate whether genistein stimulated Cl^- secretion via estrogen receptors, we compared the acute effects of genistein with that of 17β -estradiol and examined the effect of genistein in the presence of a selective estrogen receptor antagonist ICI182,780. Apical and basolateral additions of 17β -estradiol at concentration of 1, 10 and 100 nM had no effect on the I_{sc} . Moreover, pretreatment with ICI182, 780 ($1 \mu\text{M}$) did not affect the basal I_{sc} ($0.07 \pm 0.14 \mu\text{A}/\text{cm}^2$, $n=3$) and failed to inhibit the increase in the I_{sc} that was induced by genistein (Fig. 6C).

Discussion

Our study is the first evidence found that genistein stimulates Cl^- secretion in endometrial epithelial cells. The genistein-activated Cl^- secretion was supported by experiments in both intact and permeabilized monolayers. First, the genistein-induced increase in I_{sc} was almost abolished by glibenclamide, NPPB and CFTRinh-172, and partly inhibited by DIDS. These are all well-known apical Cl^- channel blockers. Second, the genistein response was blocked by bumetanide, a $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransport blocker known to inhibit Cl^- entry via the basolateral membrane. Third, the genistein response did not involve Na^+ absorption as it was insensitive to the Na^+ channel blocker amiloride. Finally, genistein increased both the apical Cl^- current and basolateral K^+ current and it is general knowledge that an increased apical Cl^- permeability coupled with an increased basolateral K^+ permeability are required to sustain a driving force for Cl^- secretion [32].

Genistein elicited concentration dependent changes in the I_{sc} and the apical Cl^- current. Genistein, at concentrations $\leq 50 \mu\text{M}$, both the I_{sc} and the apical Cl^- current were stimulated in a concentration dependent manner although both were inhibited at a high concentration

(100 μ M). The inhibitory effects of genistein (> 50 μ M) are also observed in rat distal colons and nasal epithelial tissues [11, 13]. This is possibly due to the inhibition of K^+ channels. Genistein also inhibits basolateral Ca^{2+} -activated K^+ channels in the colon [11]. In CFTR overexpressing cells, genistein produces a biphasic action on CFTR activity, i.e., stimulatory effect at low concentrations and inhibitory effect at high concentrations [22]. This study suggests that at least two binding sites are required for a direct binding of genistein to the CFTR. One at high affinity reduces the closing of the channel whereas a low affinity reduces the channel's opening [22]. In the present study, genistein, when applied at high concentration (100 μ M), reduced both the apical K^+ current and the apical Cl^- current (Fig. 5). This suggests that the inhibitory effect of genistein on the I_{sc} is likely a result of the inhibitions of both the apical Cl^- and K^+ channels.

Genistein has been reported to activate CFTR in the CFTR overexpression system, cultured cell lines and native epithelial tissues [11, 14, 17, 20, 22, 23, 36]. Some studies have shown that genistein alone activates CFTR [14, 23] whereas other studies have shown that a cAMP dependent phosphorylation of CFTR through forskolin is required for the stimulatory effect of genistein [11, 23, 36]. However, the pre-activation with cAMP was not required in the present study since genistein directly stimulated the I_{sc} . This increased I_{sc} by genistein was significantly reduced in the presence of forskolin, an activator of adenylate cyclase or IBMX, a phosphodiesterase inhibitor. The forskolin/IBMX-stimulated I_{sc} was also abolished in the presence of genistein. These findings are consistent with previous studies which have indicated that genistein shares a common Cl^- secretory pathway which is activated via cAMP-dependent agonists [14, 36], and that the genistein response is at least involved in cAMP-activated Cl^- secretion through CFTR. The participation of CFTR in the genistein response is confirmed by the observations that both, well-known and specific CFTR blockers, reduces the genistein-activated I_{sc} . Although not investigated in our study, an increase in intracellular cAMP is not required for the genistein-stimulated Cl^- secretion [19, 23]. Therefore, in the endometrial epithelial cells, genistein appears to stimulate Cl^- secretion via the direct activation of CFTR. Patch-clamp studies of heterologously expressed CFTR have reported that genistein increases the activity of CFTR by a direct interaction with NBD2 of the CFTR [17, 20].

Genistein has been reported to modulate K^+ transport in native tissues and cultured epithelial and nonepithelial cells. It inhibits basolateral Ca^{2+} -activated K^+ channels in the human rectum, the rat colon and the CFTR-expressing colonic cell line [11, 12, 14, 19]. However, it stimulates small conductance Ca^{2+} -activated K^+ channels in the collecting ducts [37]. The small conductance Ca^{2+} -activated K^+ channel mRNA is also expressed in PEG cells and is regulated by 17β -estradiol [35]. Our results demonstrated that genistein at the maximum concentration produced a biphasic response in the basolateral K^+ current. This was an initial increase in the K^+ current followed by a long-lasting decrease in the K^+ current to a persistent level below the baseline (Fig. 5C). The increased basolateral K^+ current in response to genistein was completely abolished in the presence of the non-selective K^+ channel blocker $BaCl_2$. However, the specific types of the K^+ channel in response to genistein have yet to be clarified. It is general knowledge that the activation of the basolateral K^+ channel is required to hyperpolarize the membrane producing the driving force for Cl^- secretion. Therefore, in our present study, genistein increases the I_{sc} and thus increases the Cl^- secretion by activating both the basolateral K^+ current and the apical Cl^- current. The subsequently decreased I_{sc} is likely secondary to a sustained decrease in the basolateral K^+ current. Although genistein inhibited the apical K^+ current in the current study, it seems to have less effect on the I_{sc} response when induced by genistein. Thus, the basolateral K^+ permeability appears to be the rate limiting step in respect to Cl^- secretion as has been reported previously [32].

Previous studies done on porcine endometrial epithelial cells have reported that UTP activates Cl^- secretion via an increase in the intracellular Ca^{2+} and in PKC stimulation [33, 34]. We further examined the effects of genistein in the presence of UTP. UTP rapidly increased the I_{sc} which was completely abolished in the presence of the intracellular Ca^{2+}

chelating BAPTA-AM. This confirms a dependence on intracellular Ca^{2+} mobilization and an activation of Ca^{2+} -activated Cl^- secretion by UTP. However, the increase in the I_{sc} induced by genistein was not affected by the presence of UTP. This finding suggests the possibility that genistein may stimulate Cl^- secretion directly via CFTR as has been reported by others [17, 20]. Although UTP has been shown to activate CFTR [34, 38], it is likely that the UTP-induced transient increase in I_{sc} could be a result of the stimulation of Cl^- secretion, primarily through Ca^{2+} -activated Cl^- channels. Apart from a direct interaction with CFTR, genistein may increase CFTR localization in the plasma membrane [25, 26].

In this present study, the UTP response was completely abolished in the presence of genistein. This observation indicates that genistein possibly modulates the, as yet, unknown signaling mechanisms responsible for UTP-stimulated Cl^- secretion. Some possibilities explaining the lack of UTP response in the presence of genistein are; First, genistein, a specific tyrosine kinase inhibitor, has been shown to inhibit enzymes involved in the turnover of phosphoinositol (phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate (PIP2) kinases), and hence reduces PIP2 which is the substrate for phospholipase C to produce inositol-1,4,5-triphosphate (IP3) and diacylglycerol [39]. These signaling molecules are involved in the UTP-activated Cl^- secretion in the PEG cells [33, 34]. Second, it could be explained by an inhibitory effect of genistein on the apical and basolateral K^+ currents (I_{kb}) (Fig. 5). A sustained and marked decrease following an immediate increase in the I_{kb} may prevent the stimulatory effects of UTP on the I_{sc} . The apical small-conductance Ca^{2+} -activated K^+ channels in the PEG cells are activated by UTP [35]. A reduction in K^+ secretion through K^+ channels by genistein would depolarize the membranes, thus reducing the driving force for the UTP-stimulated Cl^- secretion. Third, genistein appears to partially activate the Ca^{2+} -activated Cl^- channels since approximately 65% of the genistein response was also inhibited in the presence of DIDS (Fig. 2). Although, genistein action is independent of intracellular Ca^{2+} , the participation of the Ca^{2+} -activated Cl^- channels in the genistein response may thereby reduce a further increase in the I_{sc} upon the application of UTP. However, the exact mechanism could be the subject of further studies.

Genistein has been reported to regulate Cl^- secretion through the modulation of the phosphorylated state of CFTR by protein kinases and protein phosphatases [18, 19, 23]. Indeed, genistein has been shown to inhibit tyrosine kinase [40] and regulation of tyrosine phosphorylation has been proposed in modulating the CFTR and thus Cl^- secretion in both native tissues and cultured cells [14, 23]. In the present study, tyrphostin A23, a tyrosine kinase inhibitor that is structurally and mechanistically different from genistein reduced the basal I_{sc} and limited the genistein-increased I_{sc} . These findings resemble those observed in the previous studies with renal epithelial A6 cells, human colonic T84 cells and mouse jejunum [14, 15, 23]. However, the inhibitory effect of tyrphostin A23 on the basal I_{sc} is unknown and is suggested that it involves the direct blockade of the CFTR or inhibition of the protein kinase A as has been observed at high concentrations of the erbstatin compound [23]. The genistein response was also abolished in the presence of AG490, a tyrphostin that specifically inhibits tyrosine-specific protein kinase [41]. The different effects of tyrosine kinase inhibitors on transport function is also dependent on the structural nature of the inhibitor as has been proposed by Niisato et al. [15]. Genistein, a soy isoflavone, has a structure which is mechanistically different from erbstatin-derived tyrphostins. However, in the present study, daidzein, an isoflavone that is a structural analog of genistein, had less of a stimulatory effect on the basal I_{sc} when compared to that of genistein. This is also suggestive of the complex role of isoflavones on ion transport functions. Daidzein has been reported to have no activity or less specificity than genistein for the inhibition of protein tyrosine kinase [39]. The lack of a daidzein effect on cAMP-stimulated Cl^- secretion has been evidenced in T84 cells [23]. The reduced genistein response in the presence of tyrphostin A23 or AG490 suggests that genistein acts in the stimulation of Cl^- secretion via the inhibition of a tyrosine kinase. Additionally, pretreatment with vanadate, a tyrosine phosphatase inhibitor, failed to inhibit the stimulatory effects of genistein on the I_{sc} suggesting that the inhibition of protein tyrosine phosphatase does not involve a genistein response in the PEG cells. Similar

findings are also observed in Hi-5 insect cells and CFTR expressing NIH3T3 fibroblast cells in demonstrating the lack of a genistein effect in the presence of vanadate [22]. All of the present findings indicate that the effects of genistein on CFTR and thus, Cl^- secretion appear to be modulated by a tyrosine kinase-dependent phosphorylation pathway.

Since genistein is structurally related to estradiol, its effect is suggested to be mediated through estrogen receptors (ER) with expression on the target organs. Genistein binds to both ER- α and ER- β subtypes, with a higher affinity to ER- β when compared to endogenous estrogen [2]. Estrogen and genistein have been reported to act via the ER-mediated pathway in the regulation of protein expressions which include protein transporters and receptors. In particular, treatments of kidney BHK cells with genistein (30 μM) for a period of 24 hours has been shown to up-regulate CFTR expression [42]. The effect of genistein on protein expression appears to be modified by ligand dose, ER subtypes and gender. Al-Nakkash [43] had shown evidence on the stimulatory effects of genistein on jejunal chloride secretion in male mice via an ER-dependent mechanism and in female mice via an ER-independent mechanism. In addition, the expressions of ER α and ER β in the endometrial cells varied dependent upon the concentration of genistein [44]. On the other hand, estrogen and genistein has been shown to regulate protein functions independent of protein expression. Studies in the colonic epithelium have demonstrated that 17 β -estradiol acutely modulates the Cl^- secretory process via a membrane ER [45]. Genistein has also been reported to acutely stimulate HCO_3^- secretion in mouse duodenum, primarily through an ER-dependent pathway [46]. Contrastingly, in our study, the acute effect of genistein on Cl^- secretion is not mediated through ER as the selective estrogen receptor antagonist, ICI182,780, failed to inhibit the genistein-stimulated I_{sc} . Moreover, 17 β -estradiol did not affect the basal I_{sc} . The ER-independent acute response of genistein on chloride secretion may be of benefit to all tissues and either gender; even though the specific subtypes of estrogen receptors do not express themselves.

Any alterations in uterine volume and electrolyte composition, away from the norm, may cause an unfavorable uterine environment for many reproductive processes and may lead to infertility. In cystic fibrosis, reduced fertility is the result of a loss of CFTR-mediated anion secretions and thus, a reduction of fluid secretion from the glands into the uterine lumen. In this case, genistein may be used as pharmacological intervention for infertility that is associated with cystic fibrosis. Conversely, daily consumption of food or plants high in genistein or isoflavones has shown incidences of infertility in sheep and cheetahs [47, 48], but little information is known about its effects in humans. The cause of infertility may be the result of the accumulation of excessive fluid in the uterine lumen as has been supported by a recent study in rat uterus that has reported excessive fluid secretion and high luminal fluid volume with high genistein treatments [7]. In this study, subcutaneous injections with high dose genistein (100 mg/kg/day) for 3 days resulted in plasma levels of 15 μM which is comparable to the results of EC_{50} in the genistein response in our present study. By comparison, rats consuming a diet containing 750 $\mu\text{g/g/day}$ of genistein have been shown to generate serum genistein concentrations of 1 μM [49]. The excessive fluid accumulation in the uterine lumen may cause adverse effects on many reproductive processes including; sperm capacitation, fertilization and embryo implantation. While the observed adverse effects of genistein on the uterine fluid secretions are related to high daily or long term consumptions of genistein, our present study provides evidence on the direct action of genistein-induced fluid secretions and has demonstrated that the major component of the fluid secreted is likely Cl^- . From this information, given the acute and transient effects of genistein on Cl^- secretion, it is implied that short term or intermittent consumption of genistein or isoflavone containing diet is unlikely to interfere with the normal uterine volume and composition as well as one cause of infertility. Nevertheless, high intake or long term consumption of these isoflavones should be taken into consideration as to the adverse effects that have been found in humans and in animals.

In conclusion, the present study has demonstrated that the isoflavone genistein acutely stimulates Cl^- secretion in porcine endometrial epithelial cells by activation of apical Cl^- and basolateral K^+ conductance. The genistein-activated Cl^- secretion may involve the direct

activation of CFTR which appears to be mediated by the tyrosine-dependent phosphorylation pathway. Our study provides functional information of the action of genistein on the modulation of Cl⁻ secretion in the endometrial epithelium. The use of soy-derived isoflavone genistein may be of benefit as a pharmacotherapeutic agent for the treatment of electrolyte transport disorders in epithelial tissues.

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