Cellular Physiology and Biochemistry

Cell Physiol Biochem 2012;30:137-150
DOI: 10.1159/000339053
Published online: June 18, 2012
Accepted:May 30, 2012

1015-8987/12/0301-0137$38.00/0

Original Paper

Genistein Stimulates Jejunal Chloride Secretion via Sex-dependent, Estrogen Receptor or Adenylate Cyclase Mechanisms

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Key Words
Genistein • Estrogen receptors • Estradiol • Adenylate cyclase • Chloride secretion • Jejunum • Intestine • Signaling pathways

Abstract
Background/Aims: Daily subcutaneous injections with the phytoestrogen genistein, 600 mg/kg genistein/day (600G) significantly increased intestinal chloride (Cl^-) secretion (I_{sc}, $\mu A/cm^2$) in C57BL/6J female and male murine jejunum after 1-2-weeks treatment. ***Methods and Results:*** In 600G females, basolateral application of the adenylate cyclase inhibitor MDL-12330A (10 μM) significantly reduced basal and total I_{sc} in the presence of forskolin (27 and 40% respectively, $P < 0.05$), with no effect in 600G males, suggesting that 600G-mediated increases in I_{sc} in females are due to an adenylate cyclase-dependent mechanism. Concomitant injections with the non-selective estrogen receptor (ER) antagonist ICI-182780 (25 mg/kg/day) resulted in a significant inhibition of basal I_{sc} in males (38%, $P < 0.05$), but was without effect in females (further reinforcing an ER-independent mechanism of action). The ERα-selective antagonist (MPP, 25 mg/kg/day) similarly significantly inhibited the basal I_{sc} (37%, $P < 0.05$) in males, whereas the ERβ-selective antagonist (PHTPP, 25 mg/kg/day) was without effect, suggesting that 600G-mediated increases in I_{sc} in male mice are due to an ERα-dependent mechanism. Jejunum ERα/actin expression was significantly increased by 600G in males. Compared to intact mice, orchiectomy has differing effects on 600G-mediated basal Isc; castration (CAST) abolished the 600G-mediated increases in I_{sc}, and ovariectomy (OVX) had no effect on the 600G-stimulated increases in I_{sc}. Daily estradiol injections (10-20 mg/kg body weight estradiol (10E2 or 20E2) had no effect in intact females, whereas 10E2 significantly increased basal I_{sc} in OVX females. ***Conclusion:*** These data suggest that daily estradiol and genistein injections have differential sex-dependent mechanisms of action on murine intestinal Cl^- secretion.

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Introduction

Genistein, a naturally occurring isoflavonic phytoestrogen found in high concentrations in soy products is structurally similar to estrogen [1, 2]. Moreover, evidence suggests that genistein binds to estrogen receptors [3, 4], and has binding affinities to ERα and ERβ that are comparable to 17β-estradiol [5], although, a higher binding affinity for isoflavones to ERβ compared to ERα has also been described [6]. In general, the role of ER's in colonic tissue has commanded a greater focus compared to an examination of their role in the small intestine. Despite this, the evidence for the expression of ER's, let alone their role within the intestines is currently both contradictory and limited: i.e. a role for ERβ in the organization and architectural maintenance of the colon in mice [7]; a lack of expression of ERα in the small intestine and colon of young and old male and female rats [8], greater expression of ERα than ERβ in colonic tissue from 8-weeks old rats, along with an isoflavone-induced (765 mg/kg diet for 2-weeks) increased expression of ERα and concomitant decreased expression of ERβ [9], and, expression of both ERα and ERβ in the adult male and female murine small and large intestine [10].

Our previous evidence shows that mice fed a diet of 600 mg genistein/kg food for 4 weeks, yield serum concentrations of ~7 μM in female mice and ~4 μM in male mice [11], levels that are comparable to a soy milk diet in humans [12]. This dietary exposure to genistein (600 mg genistein/kg food for 4-weeks) stimulated basal Cl^- secretion across freshly isolated segments of jejunum from female mice, but not male mice [11]. Moreover, we have recently shown that daily genistein (600 mg genistein/kg body weight, 600G) subcutaneous (s.c.) injections for a period of 1-week in female mice, or 2-weeks in male mice, also significantly increases serum genistein levels (~5 μM and ~4 μM respectively), compared to 0G female and male controls, which had non-detectable serum genistein levels [13]. These data suggest that regardless of route of administration of genistein, diet or daily s.c. injections, serum genistein levels are measurably increased. The advantage of the use of daily s.c. injections route of genistein administration versus diet, is that it yields less variable and more sustained elevations in intestinal anion secretion, as we have shown previously [11, 13].

The manipulation of tissue function by genistein has been described in several tissues; Noel et al. [14] demonstrated that s.c. injection of 50 μM genistein (or MPB-07), in the presence of isoprenaline (10 μM), induced salivary secretion in *Cftr*$^{+/+}$ mice, and more recently, Tuo *et al.* provided evidence of genistein-stimulated increases in duodenal HCO_3^- secretion, mediated via an ER and PI3K-dependent pathway [15, 16].

We have recently described the effects of 1- or 2-weeks with daily sc. genistein injections, 600 mg/kg body weight/day (600G), or the vehicle control (0 mg/kg body weight/day, 0G), on small intestinal (jejunum) epithelial anion secretion (using freshly excised jejunum segments) in female and male mice [13]; (1) basal I_{sc} was significantly increased with daily 600G injections, after 1-week in females (215.9 ± 13.1 μA/cm^2 (n = 15) and 126.5 ± 10.1 μA/cm^2 (n = 8) respectively, $P < 0.05$) and 2-weeks in males (186.6 ± 7.6 μA/cm^2 (n = 5) and 100.8 ± 18.2 μA/cm^2 (n = 7) respectively, $P < 0.05$), (2) there was some 600G-dependent CFTR–mediated contribution towards the Cl^- secretion, and (3) there was a modest 5% increase in CFTR trafficking to the apical membrane in males (but not females).

Here, we aimed to further understand genistein's mechanism of action on Cl^- secretion in jejunum, after treatment for 1-week in females and 2-weeks in males with daily subcutaneous injection of genistein (600 mg/kg body weight/day, 600G). Pharmacological antagonists for the estrogen receptors (ER's) were used to assess whether the stimulatory action on jeunual Cl^- secretion induced by 600G, involved ERα and/or ERβ. We examined the effects of genistein in the presence and absence of endogenous sex-hormones using ovariectomized females and castrated males, and the effects of genistein and estradiol were compared. Moreover, we examined the effects of several pharmacological inhibitors

to determine intracellular signaling pathways. We hypothesized that genistein would have a gender-dependent mechanism of action.

Materials and Methods

Mice

Male and female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 4-6 weeks of age and housed in an animal care facility with 12:12-hour light-dark cycle. Mice consumed food and water ad libitum. Body weight and general health were monitored biweekly. Mice were fed a casein based genistein-free diet throughout the study and randomly assigned to one of the following injection groups; 600G (600 mg/kg body weight genistein), or 0G (0 mg/kg body weight genistein – genistein free) for either 1- or 2-weeks depending on the sex (males 2-weeks and females 1-week). Subsets of mice were assigned to receive concomitant injections with either 7a,17b-[9-[4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI-182780 – a non-selective ER antagonist, 25 mg/kg/day), 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP – an ERα antagonist, 25 mg/kg/day), or 4-[2-phenyl-5,7-bis9trifluoromethyl)pyrazolol[1,5-a]pyrimidin-3-yl]phenol (PHTPP – an ERβ antagonist, 25 mg/kg/day), for the last 5 days of the study to determine involvement of estrogen receptors. Additional groups of female mice were injected with either 10 mg/kg body weight estradiol (10E2) or 20 mg/kg body weight estradiol (20E2) to compare the effects of estradiol (E2) with those of genistein.

At the end of the injection study period, mice were asphyxiated in an atmosphere of 100% CO_2, followed by surgical thoracotomy to induce pneumothorax. Animal care and treatments were conducted in accordance with established guidelines and all protocols were approved by Midwestern University IACUC. *Diets.* The casein-based diet was prepared by Dr. R. S. MacDonald (Department of Nutrition, Iowa State University) and contained 0G and had an estimated energy content of 16.28 kJ/g. Diet composition is described previously in Al-Nakkash et al. [11].

Estrogen receptor, ERα and ERβ, western blot

At collection, segments of cleaned jejuna were immediately snap frozen in liquid nitrogen and stored at -80°C. Jejuna were later prepared for western blot analysis by homogenization. The western blot protocol was similar to that described previously [13]. Briefly, samples were analyzed for protein content, and ran on 4-12% Tris-Glycine gels at 150 V for ~ 1.5 hours (for both ERα and ERβ). Transfer was for 2 hours at 30 V on ice. Blots were incubated with primary antibody to either ERα or ERβ (1:500 and 1:250 dilutions respectively) overnight at 4°C. After washing, blots were incubated with donkey anti-mouse IgG, HRP conjugated secondary antibody (1:5000 dilution for ERα and 1:1000 for ERβ) for 1 hour at room temperature. To re-probe for actin: blots were incubated with anti-actin primary antibody (1:7500 or 1:20000 dilutions for ERα and ERβ respectively) overnight at 4°C. Blots were washed and then re-incubated with an anti-mouse IgG, HRP conjugated secondary antibody (1:15000 or 1:5500 dilutions for ERα and ERβ respectively). Blots were visualized using ECL (Amersham, Piscataway, NJ). Images were taken and analyzed using the STORM 860 scanner (Molecular Dynamics, Piscataway, NJ) and image quant (Molecular Dynamics, Piscataway, NJ).

Bioelectric measurement of intestinal secretion

Via an abdominal incision, ~5 cm of mid-jejunum was removed and placed in ice-cold oxygenated Krebs bicarbonate ringer (KBR). Each mouse yielded 2-3 jejunum pieces, isolated as described previously [11, 13, 17-19]. Jejunum intact (whole mount, un-stripped) sections mounted in the Ussing chambers had 0.3 cm^2 exposed surface area. Whole mount jejunum has been shown to respond to acute application of genistein with favorable time courses. Evidence published from Hamilton's lab [20, 21] provides support for genistein's stimulatory action on intact jejunum when applied acutely, and, as with our studies, it appears that the presence of the circular and longitudinal muscle layers surrounding the jejunum did not impede access of chemicals to the epithelium, as exhibited by rapid I_{sc} responses with the addition of forskolin or bumetanide. Transepithelial short circuit current (I_{sc}, $\mu A/cm^2$) was measured via an automatic voltage clamp (VCC-600, Physiologic Instruments, San Diego, CA) and the experimental conditions and methods were as previously described [22]. Intestinal tissue pieces were maintained in 1 μM indomethacin (minimizing tissue exposure to endogenously generated prostanoids due to manipulation and mounting of the tissue, [23]. Glucose (10 mM) was added to the serosal KBR bath and mannitol (10 mM) substituted for glucose in the mucosal KBR bath, to avoid an inward current due to Na^+-coupled glucose transport [22].

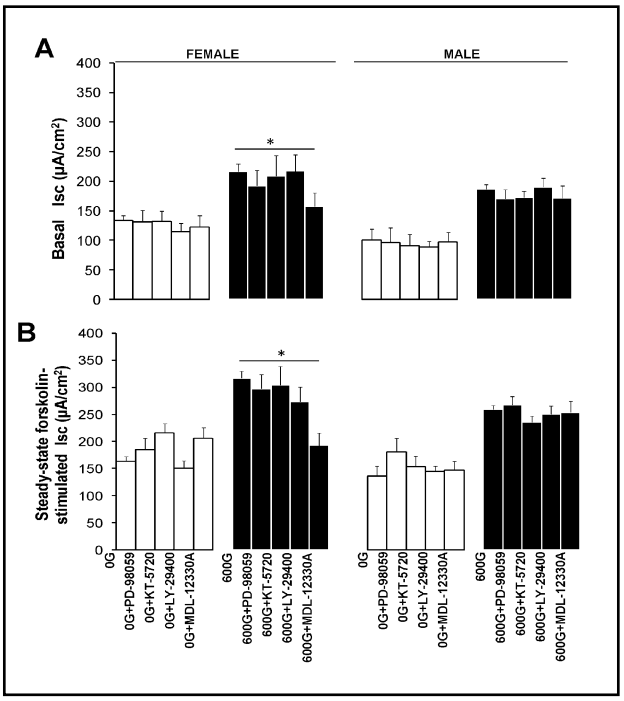

Fig. 1. Effect of signaling pathway inhibitors on basal and cAMP-stimulated I_{sc} in female and male mice. A. Average basal I_{sc} from female and male mice injected with either 600G (solid bars, n = 5-15), or 0G (open bars, n = 4-9). B. Average total I_{sc} in the presence of forskolin (10 μM, bilateral) from female and male mice injected with either 600G (solid bars, n = 5-15), or 0G (open bars, n = 4-9). Values are means ± SEM. * denotes significant inhibition from 600G alone, $P < 0.05$.

Once mounted, the serosal side was exposed to tetrodotoxin (0.1 μM), minimizing variations in intrinsic intestine neural tone [24]. Intrinsic neural tone limits the absorptive capacity of the murine mucosa (decreased I_{sc} denotes neural block). *Experimental protocols: Protocol 1:* Tissues were exposed to KBR (20 min) and steady-state basal I_{sc} measured at that time. cAMP-dependent anion secretion was assessed by bilateral application of 10 μM forskolin (at time 20 min) and total I_{sc} in the presence of forskolin was noted (at time 50 min). Glucose (10 mM, mucosal) was added at the end of each experiment to stimulate Na^+-coupled glucose transport and assess tissue viability (as denoted by > 10% increase in I_{sc}). Tissues failing to respond to glucose within this parameter were discarded. *Protocol 2:* Tissues were exposed to KBR (20 min) and steady-state basal I_{sc} measured at that time. A signaling pathway inhibitor was added (basolateral, at time 20 min): MAPK inhibitor, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD-98059, 10 μM); PKA inhibitor, (9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3, 4i][1, 6]benzodiazocine-10-carboxylic acid hexyl ester (KT-5720, 1 μM); PI3K inhibitor, 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY-294002, 20 μM); adenylate cyclase inhibitor, *cis*-N-(2-Phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL-12330A, 10 μM), and its effect noted at time 40 min. cAMP-dependent anion secretion was assessed by bilateral application of 10 μM forskolin (at time 40 min) and the steady-state forskolin response (at time 70 min). Glucose (10 mM, mucosal) was added at the end of each experiment. *Solution:* Cl^--containing KBR contained the following (in mM): 115 NaCl, 25 $NaHCO_3$, 5 KCl, 1.2 $MgCl_2$ and 1.2 $CaCl_2$, pH 7.4.

Chemicals

Forskolin was purchased from Calbiochem (San Diego, CA). ICI-182780, MPP and PHTPP were purchased from Tocris Bioscience (Ellisville, MO). PD-98059, KT-5720, LY-294002, and MDL-12330A were all purchased from Sigma Aldrich (St Louis, MO). Western blot antibodies: ERα and ERβ (Santa Cruz Biotechnology Inc, CA), donkey anti-mouse IgG, HRP conjugated (Santa Cruz Biotechnology Inc, CA), Anti-Actin (Millipore, Temecula, CA) and anti-mouse IgG, HRP conjugated (Upstate/Millipore, Temecula, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

Data are expressed as mean ± SEM. Numbers in parentheses are numbers of tissues used from separate individual mice. One-way ANOVA with Neuman- Keul's multiple comparison test or t-tests were performed using GraphPad (San Diego, CA). $P < 0.05$ was considered statistically significant.

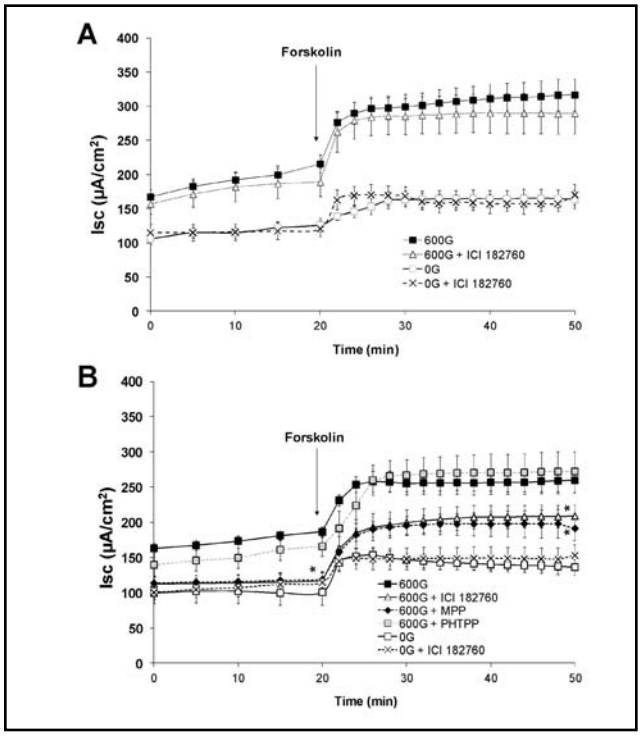

Fig. 2. Effect of estrogen receptor antagonists on basal and cAMP-stimulated I_{sc} in female and male mice. A. Average basal and total I_{sc} in the presence of forskolin (10 μM, bilateral) from female mice injected for 1-week with either 600G (solid squares, n = 11), 600G+ICI 182780 (open triangles, n = 5), 0G (open squares, n = 8) and 0G+ICI 182760 (-X-, n = 7). B. Average basal and total I_{sc} in the presence of forskolin (10 μM, bilateral) from male mice injected for 2-weeks with either 600G (solid squares, n = 5), 600G+ICI 182780 (open triangles, n = 6), 600G+MPP (solid diamonds, n = 5), 600G+PHTPP (gray squares, n = 8), 0G (open squares, n = 7) and 0G+ICI 182760 (-X-, n = 5). Values are means ± SEM. * denotes significant inhibition from same time point with 600G alone, $P < 0.05$.

Results

Effect of signaling pathway inhibitors on intestinal Isc

We examined the effect of four signaling pathway inhibitors on basal and total I_{sc} in the presence of forskolin, in male and female mice injected with either 600G or 0G (Fig. 1). There was no effect of the basolateral addition of the MAPK inhibitor PD-98059 (10 μM), PKA inhibitor KT-5720 (1 μM) or the PI3K inhibitor LY-294002 (20 μM) on basal I_{sc} or total I_{sc} in the presence of forskolin, in males or females injected with either 0G or 600G.

In 600G females, the adenylate cyclase inhibitor MDL-12330A (10 μM) significantly inhibited both the basal I_{sc} (by 27%, $P < 0.05$) and total I_{sc} in the presence of forskolin (by 40%, $P < 0.05$, Fig. 1A, B). No effect of MDL-12330A was observed in the other groups. These data suggest that the adenylate cyclase signaling pathway is involved in the genistein-mediated increases in anion transport in females, but does not have a role in the 600G-stimulated increase in intestinal secretion in males.

Effect of ER antagonists on intestinal Isc

Since genistein is structurally similar to estrogen, we aimed to determine the role of ER's in mediating the 600G-stimulated increases in basal and total I_{sc} in the presence of forskolin. In female mice, concomitant injections of the non-specific ER antagonist ICI-182780 (25 mg/kg/day) with 0G or 600G injections, had no effect on either the basal I_{sc} or the total I_{sc} in the presence of forskolin, compared to 0G or 600G alone (Fig. 2A). These data suggest that genistein-mediated increases in basal and total I_{sc} in the presence of forskolin in female mice are not mediated via an ER-mediated pathway.

Male 600G mice concomitantly injected with the non-specific ER antagonist ICI-182780 significantly decreased the basal I_{sc} compared to 600G alone (by 38%, $P < 0.05$, Fig. 2B) and decreased the total I_{sc} in the presence of forskolin compared to 600G alone (by 20%, $P < 0.05$, Fig. 2B). There was no effect of ICI-182780 in 0G males (Fig. 2B). These data suggest that in males, 600G-mediated increases in basal and total I_{sc} in the presence of forskolin are mediated via an ER-dependent pathway.

To further elucidate which ER's were responsible, we concomitantly injected two additional subgroups of male 600G mice with either an ERα-selective antagonist (MPP, 25

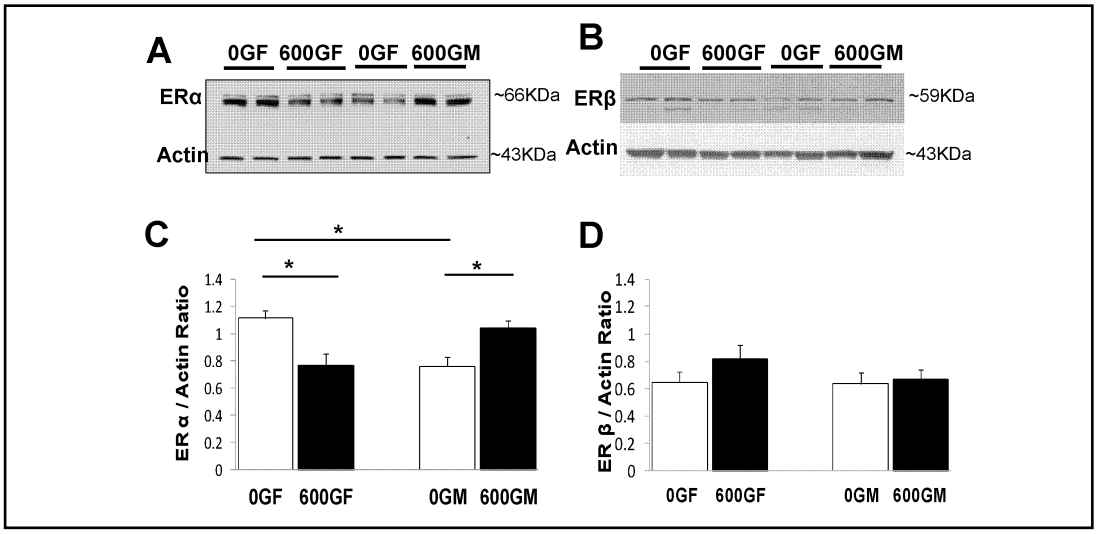

Fig. 3. Effect of genistein treatment on total ERα and ERβ protein expression in female and male murine jejunum. A. Typical western blot demonstrating ERα and actin expression in jejunum from 2 samples each of 600G and 0G treated female and male mice. ERα and actin bands were observed at ~66 KDa and 43KDa respectively. B. Typical western blot demonstrating ERβ and actin expression in jejunum from 2 samples each of 600G and 0G treated female and male mice. ERβ and actin bands were observed at ~59 KDa and 43KDa respectively. C. Average ERα/actin ratio comparing 600G (solid bars) and 0G (open bars) treated male and female mice (n = 11/group). D. Average ERβ/actin ratio comparing 600G (solid bars) and 0G (open bars) treated male and female mice (n = 6-8/group). Values are means ± SEM. * denotes significance, $P < 0.05$.

mg/kg/day) or an ERβ-selective antagonist (PHTPP, 25 mg/kg/day). Co-injection with MPP and 600G significantly decreased all I_{sc}'s compared to 600G alone; basal I_{sc} decreased by 37% ($P < 0.05$) and total I_{sc} in the presence of forskolin decreased by 27% ($P < 0.05$, Fig 2B). There was no effect of co-injection with PHTPP (Fig 2B). These data suggest that 600G-mediated increases in basal and total I_{sc} in the presence of forskolin in male mice are mediated via an ERα-dependent pathway.

Effect of genistein on intestinal ERα and ERβ protein expression

Since our transepithelial short circuit current measures using ER antagonists suggested that genistein-mediated increases in basal and total I_{sc} in the presence of forskolin were mediated in male mice (but not females) via an ERα-dependent pathway, we next aimed to determine whether or not ERα and/or ERβ expression was increased in males versus females, and/or changed by the genistein treatment. Standard western blot techniques were utilized to determine total jejunal ERα or ERβ expression (at ~66 or ~59 KDa respectively), normalized to actin (~43 KDa). ERα/actin expression was significantly decreased in 0G males (by 32%, $P < 0.05$) compared to 0G females; suggesting a gender-dependent difference under resting control conditions (Fig. 3A, C). Moreover, genistein treatment (600G) had differing effects on ERα expression; 600G significantly decreased ERα expression in females by ~31% (n = 11, $P < 0.05$), whereas 600G significantly increased ERα expression in males by ~27% (n = 11, $P < 0.05$, Fig. 3A, C). These data suggest that ERα expression is differentially regulated in male and female mice by genistein. There was no effect of either sex or genistein-treatment on ERβ expression in jejunum (Fig. 3B, D).

Effect of genistein and orchiectomy on intestinal Isc

We compared the effect of 600G on intact mice versus those with orchiectomy (ovariectomized (OVX) females, or castrated (CAST) males). Both intact and OVX females

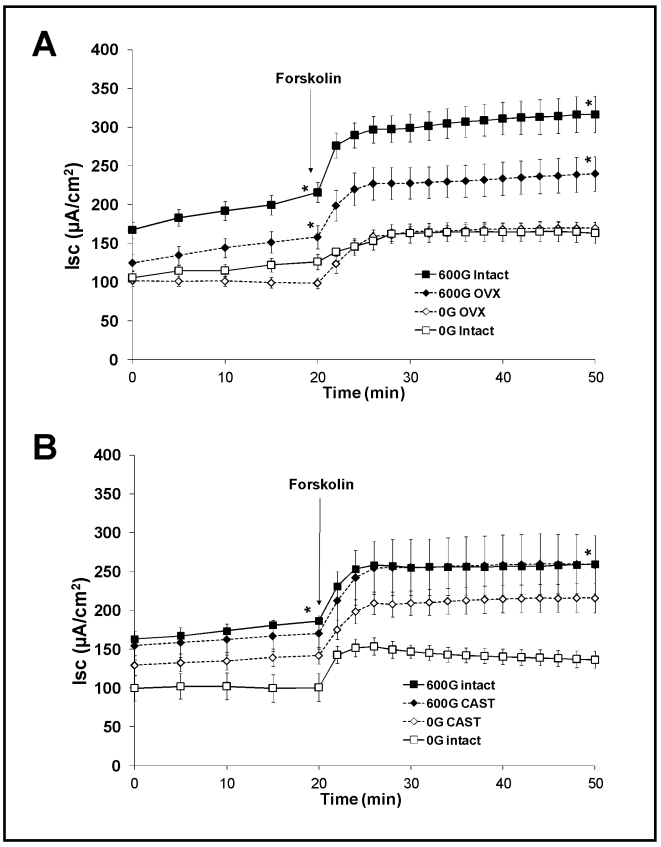

Fig. 4. Effect of 600G on average basal and cAMP-stimulated I_{sc} in intact and orchiectomized female and male mice. A. A comparison of the basal and total I_{sc} in the presence of forskolin (10 μM, bilateral) responses from female mice injected for 1-week with either 600G; intact (filled squares, n = 15), OVX (filled diamonds, n = 14), or 0G; intact (open squares, n = 8), OVX (open diamonds, n = 10). B. A comparison of the basal and total I_{sc} in the presence of forskolin (10 μM, bilateral) responses from male mice injected for 2-weeks with either 600G; intact (filled squares, n = 5), CAST (filled diamonds, n = 5), or 0G; intact (open squares, n = 5), CAST (open diamonds, n = 7). Values are mean ± SEM, * denotes significant difference from 0G, $P < 0.05$.

Parameter	Group	0G	600G
Basal	Intact Female	126.5±10.1 (8)	215.9±13.1 (15)*
	OVX Female	96.8±6.8 (10)	158.9±15.2 (14)*#
Basal	Intact Male	100.8±18.2 (7)	186.6±7.6 (5)*
	CAST Male	141.9±11.1 (5)#	170.5±20.4 (5)
SS-Fsk	Intact Female	163.4±13.6 (8)	316.6±23.4 (15)*
	OVX Female	169.9±7.9 (10)	240.5±22.3 (14)*#
SS-Fsk	Intact Male	136.5±10.9 (7)	259.8±37.8 (5)*
	CAST Male	215.9±18.5 (5)	259.1±37.8 (5)

Table 1. Effect of orchiectomy and genistein on Isc:The following basal and total Isc in the presence of forskolin (SS-Fsk) Isc (μA/cm2) measures were taken in both female and male mice (ovariectomized (OVX), castrated (CAST), or intact), injected with 600G (genistein-treated), or 0G (genistein-free). Data is expressed as mean ± SEM (numbers in parentheses are numbers of animals). * denotes significant difference to 0G ($P < 0.05$), # denotes significant difference to intact counterparts ($P < 0.05$).

injected with 600G had significantly increased basal I_{sc} (by ~1.6-fold, $P < 0.05$) and elevated total I_{sc} in the presence of forskolin (by ~1.4-2.0-fold, $P < 0.05$) compared to their 0G controls (Fig. 4, Table 1). Intact females responded with a significantly greater increase in both basal I_{sc} (by 1.4-fold, $P < 0.05$) and total I_{sc} in the presence of forskolin (by 1.3-fold, $P < 0.05$) compared to the OVX females (Fig. 4A, Table1). Basal I_{sc} was similar for both 0G controls in intact and OVX groups (Fig. 4A, Table 1).

Only intact 600G males had a significantly increased basal I_{sc} (by 1.9-fold, $P < 0.05$) and total I_{sc} in the presence of forskolin (by 1.8-fold, $P < 0.05$) compared to their respective 0G controls (Fig. 4B, Table 1). There was no effect of 600G in CAST males when compared

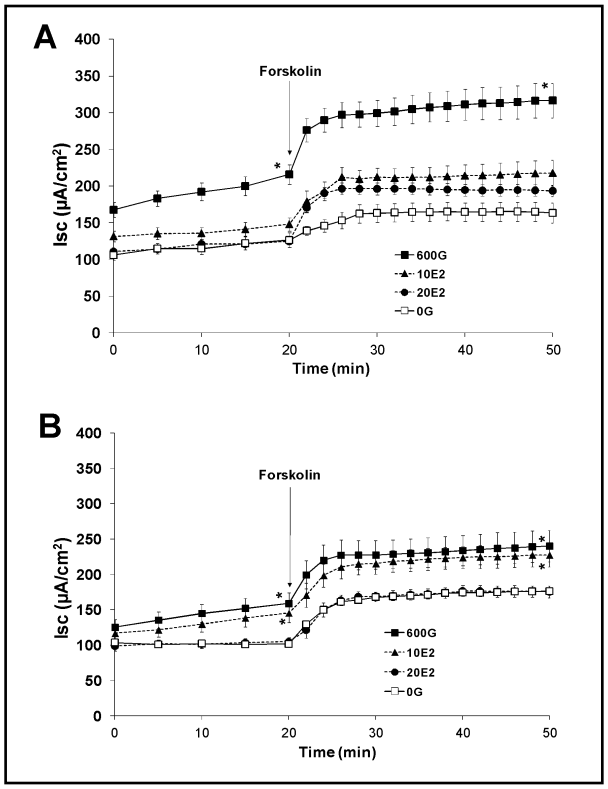

Fig. 5. Effect of 600G and estradiol on average basal and cAMP-stimulated I_{sc} in intact and orchiectomized female mice. A. Comparison of the basal and total I_{sc} in the presence of forskolin (10 μM, bilateral) responses from intact female mice injected for 1-week with either 600G (filled squares, n = 15), 10E2 (filled triangles, n = 8), 20E2 (filled circles, n = 7), or 0G (open squares, n = 8). B. Comparison of the basal and total I_{sc} in the presence of forskolin (10 μM, bilateral) responses from OVX female mice injected for 1-week with either 600G (filled squares, n = 14), 10E2 (filled triangles, n = 10), 20E2 (filled circles, n = 10), or 0G (open squares, n = 12). Values are mean ± SEM, * denotes significant difference from 0G, $P < 0.05$.

to their 0G controls, and, of note, basal I_{sc} was significantly increased in 0G CAST males compared to 0G intact males (Fig. 4B, Table1). These data suggest that orchiectomy, i.e. the absence of endogenous sex hormones, changes the responsiveness of male and female mice to 600G; OVX females respond similarly to intact females (OVX mimics the intact 600G-mediated increase in I_{sc}), whereas CAST males do not respond similarly to intact males (CAST eliminates the 600G-mediated increase in I_{sc}).

Effect of genistein and estradiol on intestinal Isc.

Given the estrogen-like characteristics of genistein (i.e. structurally similar to estrogen), we compared the effect of two concentrations of daily estradiol injections with 600G on female intact and OVX mice. In intact females, neither 10E2 nor 20E2 had an effect on basal I_{sc}, and both mimicked 0G controls, when compared to 600G (Fig. 5A). The total I_{sc} in the presence of forskolin in intact females with 10E2 or 20E2 was significantly less than with 600G (by 32% and 39% respectively, $P < 0.05$, Fig. 5A).

In OVX females, 10E2 mimicked 600G by significantly increasing basal I_{sc} (1.5-, and 1.6-fold ($P < 0.05$) respectively, Fig. 5B), compared to 0G controls and 20E2. The total I_{sc} in the presence of forskolin in OVX females with 20E2 was significantly less than with 600G (reduced by 28%, Fig. 5B). The total I_{sc} in the presence of forskolin in OVX females with 10E2 was as significantly increased as with 600G (Fig. 5B). These data suggest that in the absence of endogenous estrogen (i.e. OVX females), 10E2 and 600G act similarly to increase intestinal basal and total I_{sc} in the presence of forskolin-mediated Cl^- secretion. However, in comparison, a higher dose of E2 (i.e. 20E2) has no effect on basal and total I_{sc} in the presence of forskolin.

Discussion

Flavonoids (such as genistein) are found naturally in soy and plants and are digested in an average daily diet. Soy-rich diets have been shown to generate micromolar serum

genistein concentrations in humans [25]. Serum genistein concentrations of 1 μmol/L can be obtained in rats consuming a diet containing 750 μg genistein/g/day [26]. More recently, Bhandari et al. [27], showed a relationship between dietary genistein and serum levels in mice (1000 mg/kg dietary genistein generated ~1.5 μmol/L serum genistein and 500 mg/kg dietary genistein generated ~0.5 μmol/L serum genistein) after four weeks on their respective diets. We have previously demonstrated that daily sc. administration of genistein (600G) for a period of 1- or 2-weeks in mice induces significant increases in Cl^- secretion in freshly isolated jejuna segments [13]; 600G increases basal I_{sc} (compared to 0G) in female mice after 1-week (~82 μA/cm^2, n = 15, $P < 0.01$) and in male mice after 2-weeks (~86 μA/cm^2, n = 5, $P < 0.01$). These increases in I_{sc} were associated with concomitant increases in serum genistein levels; significantly increased in 600G females (1-week = 8.8 ± 2.1 μM (n = 5), $P < 0.05$) and 600G males (2-weeks = 4.2 ± 0.5 μM (n = 4), $P < 0.05$) compared to their respective 0G controls.

The intracellular signaling pathways involved in mediating these genistein-stimulated increases in jejunal secretion remain unknown. Recently, Tuo et al. [16] provided convincing evidence to demonstrate that acute serosal application of genistein (50 μM) stimulated duodenal HCO_3^- secretion, primarily through an estrogen receptor PI3K-dependent pathway, as indicated by the significant inhibition with either the PI3K inhibitor (LY294002, 20 μM) or the non-specific ER antagonist (ICI182,780, 10 μM). Within the intestinal epithelium, cAMP and adenylate cyclase have long been described as important regulators of ion transport (secretion) in rodent models and intestinal epithelial cell systems [28, 29]. Multiple isoforms of adenylyl cyclase have been reported in intestinal epithelium, moreover, their expression and regulation is modified via various signaling pathways including nitric oxide, in colonic epithelium [30, 31].

Regulation of intestinal transport by female sex hormones has also been described. Singh et al. [32] reported that 17β-estradiol (E2) caused a rapid and reversible inhibition of forskolin-stimulated chloride secretion across T84 epithelial cell monolayers. The difference in response by E2 on forskolin-stimulated chloride secretion in T84 monolayers, and our observations with genistein injections could be due to; (i) they used a concentration of forskolin (10 μM) which generally elicits a maximal CFTR-mediated current [33] which is not further potentiated, (ii) it is likely that E2 (like genistein [34]) has a biphasic stimulatory and inhibitory effect, and (iii) the cell systems used are different and thus may likely involve alternative pathways in either system. Additionally, female hormones, progesterone and estradiol, have been shown to inhibit CFTR-mediated ion transport in PANC-1 pancreatic epithelial cells [35], and inhibitory effects of progesterone have been shown on cAMP-mediated chloride transport in T84 intestinal cells [36].

Gender-dependent effects of E2 have been reported in rat distal colon, whereby E2 was observed to increase free intracellular Ca^{2+}, mediated via PKC and PKA, in isolated female (but not male) rat colonic crypts [37]. These gender-dependent effects of E2 were subsequently linked to a non-genomic inhibition of female (but not male) rat colonic Cl^- secretion mediated via Ca^{2+} and PKC activation [38]. Condliffe et al. [38] hypothesized that the inhibitory action of E2 on female rat distal colonic Cl^- secretion was likely mediated via inhibition of basolateral K_{Ca} and /or CFTR channel activity, and may likely be responsible for E2-induced water and salt retention.

Phytoestrogens such as genistein play an ever evolving role in intestinal transport. Genistein has been shown to rapidly and reversibly inhibit carbachol-induced colonic activity in human colonic segments mediated via ERβ and to involve p38/mitogen activated protein kinase mediated induction of nitric oxide [39]. Acute application of genistein has been shown to stimulate electrogenic Cl^- secretion across male murine jejunum [20]. The present study measures the effects of genistein after its administration *in vivo*. These data indicate that the effects of genistein must have persisted *in vitro* (for at least the experimental duration of 1.5 hours), since genistein was not administered during the measures of I_{sc}. Given the evidence

from the literature and our own previous studies demonstrating genistein's action on CFTR *in vitro* [34, 40], we previously predicted that at least one pathway that could be involved in mediating genistein's stimulatory action on the I_{sc} would be a potentiative action on the CFTR Cl^- channel itself; either increasing channel activity or channel localization, or perhaps both [13, 41]. Our recent findings suggest that genistein-mediated increases in basal I_{sc} can be attributed to a modest 5% concomitant increase CFTR localization in male jejunum (not females), suggesting that other mechanisms are playing more important roles in delivering these genistein-mediated increases in basal I_{sc} in mice [13].

The role of endogenous and exogenously applied sex hormones and/or genistein on intestinal function remains ambiguous. Thus, this study aimed to ascertain the following; (1) which intracellular signaling pathways are mediating genistein's stimulatory effects on basal and forskolin-stimulated I_{sc}, (2) whether genistein and estradiol injections have comparable effects on intestinal chloride secretion, and (3) whether the absence of endogenous sex hormones modifies the effects of genistein and estradiol on intestinal function.

Genistein is capable of binding to estrogen receptors (ER), ERα and ERβ [42, 43], both of which are found in the intestine [44, 45]. Thus, sex-dependent differences in intestinal epithelial function could be predicted if (i) genistein were to act via an ER-mediated pathway, and/or (ii) there were differences in the ER types/numbers present in male and female murine intestine. Gender differences have been demonstrated with regards to the colonic expression of ERα; reduced in males compared to female rats [46]. Those previously reported findings fit with our current observation that under control conditions (0G) female mice have significantly increased ERα (normalized to actin) expression compared to their male counterparts, but have similar ERβ expression. Manipulation of protein expression by genistein and 17β-estradiol treatment has been established in other cell systems. First, pretreatment of baby hamster kidney cells with genistein (30 μm) for 24 hours augmented CFTR maturation and localization to the cell surface [47]. Second, treatment of human bronchiolar epithelial cells, CFBE41o(-), with 17β-estradiol for 6-12 hours increased functional expression of ΔF508-CFTR, mediated via an increase in Na^+/H^+-exchanger regulatory factor 1 (NHERF1) expression [48]. Conversely, genistein has been shown to have stimulatory effects on protein function in the absence of changes in protein expression, for example: genistein has been shown to have little effect on P-glycoprotein expression whilst significantly stimulating P-glycoprotein-mediated efflux in colon carcinoma LS-180V cells [49].

Flavonoids have been reported to modify gene expression. Genistein has been shown to up-regulate expression of genes either through the estrogen response element (ERE-dependent mechanisms) or via ERE-independent mechanisms. Genistein has been documented to increase metallothionein expression in the human intestinal cells, Caco-2, and expression persisted for at least 24 hours following removal of genistein from the cell culture medium [50, 51]. It is likely that metallothionein expression in that Caco-2 cell system is regulated by other factors; trace minerals, cytokines, glucocorticoid and oxidative stress, and not ERE-dependent mechanisms [51]. In osteoblastic cells, genistein is proposed to modulate bone remodeling through ER's via a mechanism involving regulation of target gene expression, via decreased cAMP regulatory element transcription [52]. Interestingly, Chang et al. [53] provided evidence that genistein is a ligand for gene expression regulation by either ERα or ERβ, and that the gene expression is modified by ligand dose, and the ability of the ligand ER complexes to enlist various coregulators at ER binding sites. Future studies will determine whether our observed genistein-mediated ERα-dependent effects (on the I_{sc} in male mice), are mediated via ERE-dependent or –independent mechanisms.

Gender-related differences in physiology and therefore pathophysiology have been a growing focus of interest in recent years. Differences in protein expression levels in male versus female counterparts have been documented in the following studies. Female human hearts have reduced expression of a number of repolarizing K^+ channel subunits (Kv.14,

kir2.3, Kir6.2 etc), with potentially important roles in susceptibility to cardiac arrhythmias [54]. We have recently demonstrated a gender-dependent difference in jejunum epithelial basolateral K^+ channels (known to be involved in recycling of K^+, and essential to establish an optimal electrical potential for Cl^- secretion); K_{Ca}-mediated basal I_{sc} was greater in 0G males than 0G females by 2-fold, indicating a gender effect whereby K_{Ca} may play a more important role in intestinal secretion in males versus females. Similar gender-dependent differences in ion channel contribution towards tissue function have been described by other investigators in other tissues, e.g. a role for voltage-dependent K^+ channels in adenosine–mediated relaxation of coronary arterioles from male (not female) swine [55]. Inhibitory effects of 17β-estradiol on female (not male) rat distal colonic crypts have been postulated to be mediated by inhibition of basolateral K^+ channels, KCNQ1, via PKCδ- and PKA-dependent pathways [46, 56, 57]. It has been postulated that this anti-secretory response to estrogen may be representative of whole body salt and water retention during specific parts of the menstrual cycle. Whilst we did not quantify stool "fluidity" (i.e. water content) in this study, it is certainly of interest for future studies.

The cellular pathways responsible for generating the genistein- or estradiol-mediated increases in basal I_{sc} remain unclear. We show here that genistein (600G) appears to act via stimulation of adenylate cyclase in female mice, and ERα in male mice. Furthermore, removal of endogenous sex hormones (i.e. orchiectomy) produces differential effects in both sexes. The response to both intact and OVX females to 600G was a 1.7-fold increase in basal I_{sc}, whereas the response to 600G in males was not maintained in the CAST group (i.e. no effect) compared to the intact group (1.9-fold increase). These data suggest a gender-dependent requirement for endogenous sex hormones for maximal 600G-mediated increases in basal I_{sc} (not essential for females, but essential for males).

This study provides evidence that s.c. injections of genistein (600G) increase basal (and total I_{sc} in the presence of forskolin) intestinal secretion, utilizing sex-dependent mechanisms of action. Whilst the complete mechanism(s) of action of genistein mediating this effect are unclear, we can attribute the majority of this effect to an activation of ERα (in males) and adenylate cyclase (in females), which contributes towards the increase in the genistein-activated I_{sc} in both sexes. Whilst this work furthers our understanding of the role and mechanism(s) of action of genistein on jejuna I_{sc}, future studies will further elucidate the intracellular mechanism(s) involved in murine male and female genistein-stimulated jejunal secretion.

Acknowledgements

The author would like to thank Lyn Batia, Amity Peterson and Lana Leung (Midwestern University) for technical assistance, Dr. R. MacDonald (Iowa State University, Department of Nutrition) and Dr. T Broderick (Midwestern University) for helpful discussion. This work was supported by the National Institutes of Health [Grant R15 DK071625-01A2 to L.A.].

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