Dietary Genistein Rescues Reduced Basal Chloride Secretion in Diabetic Jejunum via Sex-Dependent Mechanisms

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Key Words
Ob/Ob • Genistein • Intestinal • Secretion • Transport

Abstract

Background/Aims: The goal of this study was to determine the effect of dietary genistein (naturally occurring phytoestrogen) on jejunal secretory function in a clinically relevant model of diabetes and obesity, the leptin-deficient ob/ob mouse. Methods: We measured transepithelial short circuit current (Isc), across freshly isolated segments of jejunum from 12-week old male and female ob/ob and lean C57Bl/6J mice fed a genistein diet (600 mg genistein/kg diet) for 4-weeks. Separate segments of jejunum were frozen for western blot determination of key proteins involved in secretory transport. Results: Basal Isc was significantly decreased (by 33%, P<0.05) in ob/ob females versus leans, and genistein-diet reversed this. Similarly, in males, basal Isc was decreased (by 47%, P<0.05) in ob/ob mice versus leans, and genistein-diet reversed this. Inhibition with either clotrimazole (100 µM, bilateral) or ouabain (100 µM, basolateral) was significantly reduced in ob/ob mice compared to leans (P<0.05), and genistein-diet reversed clotrimazole-sensitive inhibition in ob/ob females, and reversed the ouabain-sensitive inhibition in males (indicating sex-dependent mechanisms). Our data suggested that PDE3 levels were dysregulated in ob/ob females and genistein reversed this. Expression of total CFTR (normalized to actin) was significantly decreased ~80% (P<0.05) in all ob/ob mice compared to leans, and genistein-diet was without effect. Expression of total NKCC1 (normalized to actin) was significantly decreased ~80% (P<0.05) in ob/ob male mice versus leans, and genistein-diet reversed this. Conclusions: Our data suggests that the reduced basal jejunal Isc in ob/ob female mice is a consequence of reduced CFTR expression, decreased activities of the basolateral Kca channel and Na+/K+-ATPase, and in male mice reduced basal jejunal Isc is a consequence of reduced CFTR and NKCC1 expression, along with decreased activities of the basolateral Kca channel and Na+/K+-ATPase. Genistein-diet has beneficial effects on basal Isc mediated by sex-dependent mechanisms in diabetic mice: in females via increased Kca-sensitive Isc and in males via increased Na+/K+-ATPase activity and increased NKCC1 expression. Improved understanding of intestinal dysfunctions in the ob/ob jejunum, may allow for the development of novel drug targets to treat obesity and diabetes, and may also be of benefit in CF-related diabetes.
Introduction

The leptin deficient (ob/ob) mouse is a commonly utilized animal model for studies aimed at understanding type 2 diabetes and obesity [1]. The phenotype exhibited by this murine model closely mimics the gastrointestinal dysfunction seen clinically, including both slower gastrointestinal transit and gastric emptying time [2]. In addition to alterations in leptin function inherent to the ob/ob mouse, disturbances in intestinal monosaccharide transport appears to also play a role in developing obesity and the diabetic state, purportedly via increased fructose absorption [3] mediated via GLUT transporters [4].

The small intestinal crypts are the site of Cl secretion; Cl enters the epithelial cells via the Na+/K+/2Cl- (NKCC1) co-transporter, and there is activation of both apical Cl channels and basolateral K+ channels. A driving force for Cl exit across the apical membrane is maintained by recycling of K+ across the basolateral membrane. In addition, the Na+/K+-ATPase maintains Na+ and K+ concentration gradients across the membrane. The major route for Cl exit across the apical membrane in the normal murine intestine is via the cystic fibrosis transmembrane conductance regulatory protein, CFTR, Cl channel [5-7]. In the ob/ob mouse jejunum, the role of CFTR and the contribution of key intestinal epithelial transporters towards chloride secretion function remains relatively unclear. Aside from our initial published findings [8], indicating a reduced basal jejunal Isc in ob/ob mouse jejunum (due to reductions in: CFTR expression, activity of the basolateral K+ channel and Na+/K+-ATPase) there have been no further studies determining the relevance of a reduced Cl secretory function to the ob/ob mouse, nor any studies aimed at improving this deficiency.

Genistein, a naturally occurring (found in soy) phytoestrogen [9], is a widely recognized CFTR activator, both in isolated cells [10, 11] and tissues [12-14]. We have previously shown that C57BL/6J lean mice fed a genistein-containing diet (600 mg genistein/kg diet) for 4-weeks yield serum genistein levels of ~4-7 µM in male and female mice [13]. Such serum genistein levels are comparable to humans consuming a soy milk diet [15]. We have previously demonstrated that dietary exposure to genistein (600 mg genistein/kg diet) for a 4-week period generated significant stimulation in basal Isc across freshly isolated segments of jejunum from female lean mice. Modification of tissue function by genistein has also been examined in duodenum, whereby genistein has been shown to stimulate increased HCO3 secretion via an ER/PI3K-mediated pathway [16, 17].

The major objective of this study was to ascertain whether dietary genistein would result in improved secretory function in this diabetic model. To that end, we investigated the Isc using several pharmacological modulators, and evaluated expression of key proteins involved in the chloride secretory function of jejunum. In this study, we provide data to suggest that jejunal basal Isc is reduced in the ob/ob mouse directly due to reductions in: CFTR expression (in both males and females), NKCC1 expression (in males), reduced activity of both basolateral K+ channel and Na+/K+-ATPase (in both males and females). We provide the first evidence to demonstrate that consuming a genistein diet (600 mg genistein/kg diet) has beneficial effects on basal Isc (returning it back to levels measured in leans) which is mediated in females by an increase in the K+-sensitive Isc, whereas in males, it is mediated by an increase in Na+/K+-ATPase activity and NKCC1 expression. Of note, the phosphodiesterase enzyme (PDE) inhibitor IBMX, had a significant inhibitory effect in ob/ob females (not in males) and genistein diet reversed this. Further evaluation with milrinone suggested PDE3 is likely dysregulated in ob/ob females, and is reversed by genistein diet.

Improved understanding of the intestinal dysfunctions in the ob/ob jejunum, may allow for the development of novel drug targets to treat the intestinal complications associated with diabetes.
Materials and Methods

Mouse model of obesity

Both sexes of ob/ob and lean C57BL/6J mice (4-5 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were randomly divided into two diet groups: standard rodent diet Harlan 5001 (std), or genistein-enriched diet (600 mg genistein/kg diet, 600G) and given food and water ad libitum. The male and female ob/ob and lean mice were housed in an animal care facility with 12:12-hour light-dark cycle. Each week, body weight and overall health was examined. At ~12 weeks of age, mice were euthanized by asphyxiation in an atmosphere of 100% CO₂ followed immediately by surgical thoracotomy (thus inducing pneumothorax). Animal care was conducted in accordance with established guidelines, and all protocols were approved by the Midwestern University Institutional Animal Care and Use Committee.

Histology and morphology

Freshly isolated pieces of jejunum were embedded and flash frozen in Optimal Cutting Temperature compound (O.C.T., Tissue-Tek, Torrance, CA). For hematoxylin and eosin (H & E) staining, frozen sliced sections (8-10 µm) of jejunum were stained with a standard protocol (as previously published, [8]). Crypt depth was measured using Axiosvision (Carl Zeiss), from images of H & E stained jejunum sections. All images were taken at 10x magnification. Averages of measurements were taken from 5 separate slices per frozen section of jejunum (i.e. per mouse) and data are presented as the average of multiple mice per group.

CFTR, NKCC1 and Na⁺/K⁺-ATPase Western blot analysis

At collection, jejunum were immediately snap frozen in liquid nitrogen and stored at -80°C. Jejunum were later prepared for western blot analysis by homogenization, and the western blot protocol used was similar to that described previously [8, 18]. Blots were incubated with primary antibody to CFTR [CF3] (1:1000, Abcam Cambridge, MA), NKCC1 (1:1000, TEFS2, generous gift from Dr. Christian Lytle, University California Riverside), and Na/K-ATPase (1:1000, Cell Signaling, Danvers, MA) overnight at 4°C. After washing, blots were incubated with secondary antibody, anti-rabbit IgG (H+L) Dylight (1:15,000, Thermo Scientific, Rockford, IL), for 1 hour at room temperature. To re-probe for actin, blots were incubated with anti-actin primary antibody (1:4000, Thermo Scientific, Rockford, IL) for 1 hour at room temperature. Blots were washed and then re-incubated with the appropriate secondary antibody anti-mouse IgG (H+L) (1:15000, Dylight, Thermo Scientific Rockford, IL). Images of membranes were taken with all proteins of interest normalized to Actin. Band density was analyzed using Odyssey-Clx (LI-COR, Lincoln, NE) and Image Studio (LI-COR, Lincoln, NE).

Bioelectric measurement of intestinal secretion

Short circuit current measures were as described previously [18, 19]. Transepithelial short circuit current (Isc, µA/cm²) of 0.3 cm² exposed surface area of jejunum was measured via an automatic voltage clamp (VCC-600, Physiologic Instruments, San Diego, CA) and the experimental conditions and methods were as previously described [18, 19]. In order to reduce the tissue exposure to endogenously generated prostanooids resulting from tissue manipulation, all tissues were maintained in 1 µM indomethacin [20]. In order to avoid an inward current due to Na⁺-coupled glucose transport glucose (10 mM) was added to the serosal KBR bath and mannitol (10 mM) substituted for glucose in the mucosal KBR bath[21]. To minimize variations in intrinsic intestinal neural tone [22] and to limit the absorptive capacity of the mucosa, the serosal side of the tissues were exposed to tetrodotoxin (0.1 µM).

Experimental protocols. Tissues were exposed to KBR (20 min) and steady-state basal Isc measured at that time. In a set of tissues we added forskolin (10 µM, bilateral) until steady-state was achieved at time 20 minutes, then bumetanide (100 µM, serosal) for 10 minutes, acetazolamide for 10 minutes (100 µM, bilateral). Alternatively, in another set of tissues following steady-state basal Isc there was addition of the following pharmacological tools for 15 minutes: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS, 200 µM, bilateral), or clotrimazole (100 µM, bilateral), or ouabain (100 µM, basolateral) were used to determine the contribution to the anion secretory component by Ca²⁺-activated Cl⁻ channels, Ca²⁺-activated K⁺ channels, and the Na⁺/K⁺-ATPase, respectively. The concentrations of DIDS, clotrimazole, bumetanide and acetazolamide were as used previously [8, 18]. The concentration of
clotrimazole used is within the range of doses used in the literature (from 10 µM in colonic T84 cells [23], to 30 µM in human rectal tissue [24], to 150 µM in murine jejunum [25]. Glucose (10 mM, mucosal) was added at the end of each experiment to stimulate Na+-coupled glucose transport, as an assessment of tissue viability (denoted by > 10% increase in Isc). Tissues failing to respond to glucose within this parameter were discarded. In a subset of experiments, jejunum segments were exposed to the following phosphodiesterase inhibitors (100 µM for 10 minutes, and 200 µM for an additional 10 minutes, bilateral): milrinone, rolipram, and 8-methoxymethyl-3-isobutyl-1-methylxanthine (IBMX).

**Solutions.** Cl−-containing KBR contained the following (in mM): 115 NaCl, 25 NaHCO3, 5 KCl, 1.2 MgCl2 and 1.2 CaCl2, pH 7.4.

**Chemicals.** DIDS was purchased from Calbiochem (San Diego, CA). Clotrimazole was purchased from MP Biomedicals (Solon, OH). Ouabain was purchased from R&D Systems (Minneapolis, MN). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

**Statistics**

Data are expressed as mean ± SEM. Numbers in parentheses represent numbers of tissues used from separate individual mice. One-way ANOVA with Neuman-Keul’s multiple comparison test was performed using GraphPad (San Diego, CA) and P < 0.05 was considered statistically significant.

**Results**

**Ussing chamber bioelectric measurements**

In order to identify the effects of dietary genistein on jejunum epithelial short circuit current (transepithelial Cl− secretion, Isc) we examined freshly excised jejunum in the absence and presence of agonist stimulation. As shown in Fig. 1A, C, basal Isc was significantly decreased in ob/ob female mice (78.5 ± 6.4 µA/cm2, n = 7, P<0.05) compared to lean controls (117.2 ± 15.9 µA/cm2, n = 7), and genistein reversed this (128.9 ± 19.8 µA/cm2, n = 8, P<0.05). Basal Isc was similarly significantly decreased in ob/ob male mice (50.5 ± 9.6 µA/cm2, n = 5, P<0.05) compared to lean controls (95.2 ± 20.4 µA/cm2, n = 4), and genistein reversed this (109.0 ± 9.4 µA/cm2 n = 7, P<0.05, Fig. 1B, C). The cAMP-stimulated Isc was assessed following application of forskolin (bilateral, 10 µM, Fig. 1A, 1B, D) and was comparable between all three groups in both sexes (despite a trend for decreased cAMP-stimulated Isc intracellularly for genistein-diet) (18.7 ± 4.7%, n = 5). These data suggest that the decreased jejunum basal Isc in the diabetic ob/ob model is reversed by dietary genistein.

To better assess the role of key epithelial ion channels and transporters that may contribute towards the basal Isc, the effects of three pharmacological inhibitors applied for a period of 15 min were examined. Application of clotrimazole (bilateral 100 µM, Fig. 2A), a Ca2+-activated K+ channel blocker [19, 26, 27], resulted in significantly less % inhibition in the ob/ob female mice (-12.1 ± 5.2%, n = 7, P<0.05) compared to lean controls (50.5 ± 9.6 µA/cm2, n = 5, P<0.05) compared to lean controls (95.2 ± 20.4 µA/cm2, n = 4), and genistein reversed this (109.0 ± 9.4 µA/cm2 n = 7, P<0.05, Fig. 1B, C). The cAMP-stimulated Isc was assessed following application of forskolin (bilateral, 10 µM, Fig. 1A, 1B, D) and was comparable between all three groups in both sexes (despite a trend for decreased cAMP-stimulated Isc intracellularly for genistein-diet) (18.7 ± 4.7%, n = 5). These data suggest that the decreased jejunum basal Isc in the diabetic ob/ob model is reversed by dietary genistein.

**Ob/Ob**

In order to identify the effects of dietary genistein on jejunum epithelial short circuit current (transepithelial Cl− secretion, Isc) we examined freshly excised jejunum in the absence and presence of agonist stimulation. As shown in Fig. 1A, C, basal Isc was significantly decreased in ob/ob female mice (78.5 ± 6.4 µA/cm2, n = 7, P<0.05) compared to lean controls (117.2 ± 15.9 µA/cm2, n = 7), and genistein reversed this (128.9 ± 19.8 µA/cm2, n = 8, P<0.05). Basal Isc was similarly significantly decreased in ob/ob male mice (50.5 ± 9.6 µA/cm2, n = 5, P<0.05) compared to lean controls (95.2 ± 20.4 µA/cm2, n = 4), and genistein reversed this (109.0 ± 9.4 µA/cm2 n = 7, P<0.05) compared to those ob/ob males fed-standard chow (18.7 ± 4.7%, n = 5). These data suggest that the decreased jejunum basal Isc in the diabetic ob/ob model is reversed by dietary genistein.

To better assess the role of key epithelial ion channels and transporters that may contribute towards the basal Isc, the effects of three pharmacological inhibitors applied for a period of 15 min were examined. Application of clotrimazole (bilateral 100 µM, Fig. 2A), a Ca2+-activated K+ channel blocker [19, 26, 27], resulted in significantly less % inhibition in the ob/ob female mice (-12.1 ± 5.2%, n = 7, P<0.05) compared to lean controls (50.5 ± 9.6 µA/cm2, n = 5, P<0.05) compared to lean controls (95.2 ± 20.4 µA/cm2, n = 4), and genistein reversed this (109.0 ± 9.4 µA/cm2 n = 7, P<0.05, Fig. 1B, C). The cAMP-stimulated Isc was assessed following application of forskolin (bilateral, 10 µM, Fig. 1A, 1B, D) and was comparable between all three groups in both sexes (despite a trend for decreased cAMP-stimulated Isc intracellularly for genistein-diet) (18.7 ± 4.7%, n = 5). These data suggest that the decreased jejunum basal Isc in the diabetic ob/ob model is reversed by dietary genistein.
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**Fig. 1.** Effect of genistein on I\textsubscript{sc}.
(A) Average raw trace data of recordings from jejunum from female mice (n = 7-8/group). (B) Average raw trace data of recordings from jejunum from male mice (n = 4-7/group). Leans-standard diet (Δ), ob/ob-standard diet (○), ob/ob-genistein diet (●). Basal I\textsubscript{sc} recorded from time 0-20 mins, forskolin (10 µM, bilateral) added at time 20 mins, bumetanide (100 µM, basolateral) added at 40 mins, acetazolamide (100 µM, bilateral) added at time 50 mins and glucose (10 mM, bilateral) added at time 60 mins. (C) Average basal I\textsubscript{sc} (D) Average increase in I\textsubscript{sc} with forskolin (10 µM, bilateral). (E) Average inhibition in I\textsubscript{sc} with bumetanide (100 µM, basolateral). (F) Average inhibition in I\textsubscript{sc} with acetazolamide (100 µM, bilateral). Values are means ± SEM (n = 4-8/group). * denotes P < 0.05, statistical difference to lean controls, and # denotes P < 0.05, statistical genistein-mediated effect.

ob/ob female mice (-0.7 ± 6.2%, n = 7, P<0.05) compared to lean controls (30.9 ± 9.1%, n = 6), and was without effect by genistein-diet (6.1 ± 5.0%, n = 8). Ouabain application in males resulted in significantly less % inhibition of the basal I\textsubscript{sc} in ob/ob male mice (3.9 ± 4.1%, n = 6, P<0.05) compared to lean controls (13.0 ± 2.5%, n = 12), and was reversed by genistein-diet (45.4 ± 12.6%, n = 6, Fig. 2B). These data suggest that the deficit in Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel activity noted in female and male leptin-deficient mice is reversed by genistein-diet in females only (not males), however the deficit in Na\textsuperscript{+}/K\textsuperscript{+}-ATPase inhibition noted in both female and male ob/ob mice is reversed by genistein-diet in males only (not females).

CFTR activation is via a cAMP-dependent pathway, and cellular levels of cAMP are controlled by a balance of cAMP production (via adenylate cyclase), and degradation (via phosphodiesterases, PDE's). Therefore, we examined whether disturbances in the activities of PDE's (via hydrolysis of cAMP and/or cGMP) could contribute towards either the observed decrease in basal I\textsubscript{sc} in ob/ob mouse jejunum, or the genistein-mediated increase in I\textsubscript{sc}. We examined the effects of various isoforms of PDE's: IBMX, EHNA, milrinone, and rolipram. The non-specific PDE, IBMX (bilateral 200 µM) significantly increased basal I\textsubscript{sc} in jejunum from control female ob/ob mice (delta change in I\textsubscript{sc} = 84.8 ± 21.5 µA/cm\textsuperscript{2}, n = 8, P < 0.05), compared to leans, which was reversed in genistein-fed ob/ob female mice (delta change in I\textsubscript{sc} = 22.5 ± 3.7 µA/cm\textsuperscript{2}, n = 12, Fig. 3A). EHNA, a selective blocker of PDE2 (bilateral 200 µM), did not have an effect on jejunal basal I\textsubscript{sc} from female ob/ob control mice (delta change in I\textsubscript{sc} = 9.1 ± 6.7 µA/cm\textsuperscript{2}, n = 7) or male ob/ob control mice (delta change in I\textsubscript{sc} = 3.6 ± 2.6 µA/cm\textsuperscript{2}, n = 7). Neither was there an effect of EHNA on jejunal basal I\textsubscript{sc} from female genistein-treated ob/ob mice (delta change in I\textsubscript{sc} = -12.3 ± 6.8 µA/cm\textsuperscript{2}, n = 4) or male genistein-treated ob/ob mice (delta change in I\textsubscript{sc} = -1.6 ± 1.3 µA/cm\textsuperscript{2}, n = 6). Rolipram, a selective PDE4 inhibitor (bilateral 200 µM) had no effect on basal I\textsubscript{sc} in male or female ob/ob mice (Fig. 3B). Milrinone, a selective PDE3 inhibitor (bilateral 200 µM) significantly increased basal I\textsubscript{sc} in jejunum from
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Cellular Physiology and Biochemistry

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control female ob/ob mice (delta change in $I_{sc} = 65.1 \pm 16.4 \, \mu A/cm^2$, $n = 8$, $P < 0.05$), compared to leans, which was reversed in genistein-fed ob/ob female mice (delta change in $I_{sc} = 17.3 \pm 3.7 \, \mu A/cm^2$, $n = 14$, Fig. 3C). These data suggest that inhibition of a PDE3-dependent pathway plays a role in ob/ob females but not in males.

**Jejunum Morphology**

In theory, modifications in jejunum crypt dimensions, i.e. reduced crypt depth, could provide less available secretory epithelial cells, and could have partially explained the decreased basal $I_{sc}$ in ob/ob jejunum. Therefore, we determined whether effects on jejunal basal $I_{sc}$ were associated with changes in morphology. Histological sections were stained using H & E and analyzed for crypt depth. There was no change in crypt depth in the ob/ob female group ($89.11 \pm 3.76 \, \mu m$, $n = 15$) compared to lean controls ($87.36 \pm 6.62 \, \mu m$, $n = 15$) and genistein-diet was without effect ($80.13 \pm 4.09 \, \mu m$, $n = 14$). Similarly there were no changes in crypt depth in the male groups: ob/ob males ($83.83 \pm 2.92 \, \mu m$, $n = 17$) compared to lean controls ($91.26 \pm 6.16 \, \mu m$, $n = 12$) and genistein-diet was without effect ($86.71 \pm 42.99 \, \mu m$, $n = 13$). These data suggest that structural changes i.e. alterations in crypt dimensions are not responsible for either the reduced basal $I_{sc}$ noted in ob/ob mice, or the genistein-mediated rescue of this $I_{sc}$.

**Total CFTR, NKCC1 and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase protein expression**

Small intestinal Cl\textsuperscript{-} secretion at the crypts requires the following: Cl\textsuperscript{-} entrance into epithelial cells via the Na\textsuperscript{+}/K\textsuperscript{+}/2Cl\textsuperscript{-} (NKCC1) co-transporter, and activation of both apical Cl\textsuperscript{-} channels (CFTR) and basolateral K\textsuperscript{+} channels (to maintain the driving force for Cl\textsuperscript{-} exit across the apical membrane), and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (to maintain Na\textsuperscript{+} and K\textsuperscript{+} concentration gradients across the membrane). Since, CFTR chloride channels provide the major route
for chloride secretion into the jejunum lumen, we determined total CFTR protein present utilizing standard western blot techniques [18, 28-30]. We confirmed a single band with the expected molecular weight of ~170 kD for CFTR. We have had success with identifying CFTR expression with this antibody in wild type (lean) murine jejunum [18] and jejunum from ob/ob mice [8], and other studies have demonstrated successful use of this antibody to detect CFTR within airway epithelium [31]. Total CFTR protein expression normalized to actin (Fig. 4A) was significantly decreased (~80%) in the ob/ob female mice (0.07 ± 0.05, n = 6, P<0.05) compared to lean counterparts (0.49 ± 0.12, n = 8). Genistein-diet had no effect on CFTR expression in female ob/ob mice (0.11 ± 0.06, n = 7). Total CFTR protein expression normalized to actin (Fig. 4A) was significantly decreased (by a comparable ~80%) in the ob/ob male mice (0.14 ± 0.03, n = 7, P<0.05) compared to lean controls (0.74 ± 0.27, n = 8), and genistein-diet similarly had no effect (0.06 ± 0.04, n = 7).

To assess additional potential contributors towards the reduced jejunum secretory process in the ob/ob mice compared to lean counterparts, we evaluated total protein expression of NKCC1, and Na+/K+-ATPase. As shown in Fig. 4B, NKCC1 protein expression, was comparable in the ob/ob female mice (0.57 ± 0.15, n = 7) compared to their lean counterparts (0.50 ± 0.15, n = 9), and genistein-diet had no effect (0.38 ± 0.15, n = 9). NKCC1 protein expression was significantly reduced in the ob/ob male mice (0.07 ± 0.02, n = 7) compared to lean controls (0.30 ± 0.11, n = 9), and genistein-diet reversed this (0.30 ± 0.14, n = 9, P<0.05, Fig. 4B). Expression of total Na+/K+-ATPase protein was comparable in female ob/ob mice and female lean controls (0.29 ± 0.08, n = 11, and 0.18 ± 0.07, n = 10, respectively Fig. 4C), and genistein-diet was without effect (0.41 ± 0.13, n = 11). Expression of total Na+/K+-ATPase protein was comparable in ob/ob males, lean, and those ob/ob males fed genistein-diet (0.55 ± 0.16 (n = 9), 0.28 ± 0.12 (n = 9), and 0.49 ± 0.11 (n = 10) respectively Fig. 4C). These data suggest that expression of total CFTR was significantly decreased ~80% (P<0.05) in male and female ob/ob mice versus lean, and genistein-diet was without effect,
and expression of total NKCC1 (normalized to actin) was significantly decreased ~80% (P<0.05) in ob/ob male mice versus leans, and genistein-diet reversed this.

**Discussion**

In murine models of diabetes the small intestine remains an understudied region. The ob/ob mouse model is hypoleptinemic, hyperphagic, obese, with insulin resistance [32] and demonstrates intestinal complications that mimic those seen clinically (slowing of gastrointestinal transit [2], and gastroparesis [33]). This model of diabetes has been reported clinically [34]. The ob/ob mice used in this study are 12 weeks old, consistent with use in other studies: male ob/ob mice (6-15 weeks old) and male mice fed high fat diet (from 14-33 weeks) exhibit gastroparesis with continued elevated plasma glucose [35-37], and 15 week old male ob/ob mice exhibit slowed gastrointestinal transit [2, 38].

The jejunum epithelium is the major site for fluid secretion within the small intestine. We demonstrate significant deficits in ob/ob mouse jejunum that are consistent with our previously published evidence indicating decreased secretory function in this model [8]: (1) reduced small intestinal basal Isc in ob/ob mice compared to leans, (2) decreased contribution of Na+/K+-ATPase in ob/ob mice versus leans, (3) decreased contribution of KCa in ob/ob mice versus leans, and (4) decreased CFTR expression in ob/ob mice versus

**Fig. 4.** Total CFTR, NKCC1 and Na+/K+-ATPase protein expression in murine jejunum. (A) Typical western blot demonstrating CFTR expression (normalized to actin) in jejunum from ob/ob and lean mice. CFTR and actin bands were observed at 168 KDa and 43 KDa respectively. Average CFTR/actin ratio comparing lean (gray bars), ob/ob standard diet fed (open bars) and ob/ob genistein diet fed (solid bars), n = 6-8/group. (B) Typical western blot demonstrating NKCC1 expression (normalized to actin) in jejunum from ob/ob and lean mice. NKCC1 and actin bands were observed at 160 KDa and 43 KDa respectively. Average NKCC1/actin ratio comparing lean (gray bars), ob/ob standard diet fed (open bars) and ob/ob genistein diet fed (solid bars), n = 7-9/group. (C) Typical western blot demonstrating Na+/K+-ATPase expression (normalized to actin) in jejunum from ob/ob and lean mice. Na+/K+-ATPase and actin bands were observed at 100 KDa and 43 KDa respectively. Average Na+/K+-ATPase/actin ratio comparing lean (gray bars), ob/ob standard diet fed (open bars) and ob/ob genistein diet fed (solid bars), n = 9-11/group. Values are means ± SEM. * denotes P < 0.05, statistical difference to lean controls, and # denotes P < 0.05, statistical genistein-mediated effect.
leaks. Our data indicate that alterations in key epithelial transporters required for normal secretory function likely contributes towards the phenotype of this model.

A decreased crypt depth, could hypothetically provide less available secretory epithelial cells, and may have partially explained the reduced basal I\textsubscript{sc} in ob/ob mice. However, since crypt depth was comparable in ob/ob and lean, we focused on the dysfunctions in transporters involved in the generation of epithelial Cl\textsuperscript-- secretion. We demonstrate that basal I\textsubscript{sc} across the ob/ob mouse jejunum was significantly reduced in both male and female ob/ob mice, and this was accompanied by a ~6-fold loss of CFTR expression [8]). Interestingly, whilst genistein diet returned the reduced basal I\textsubscript{sc} of ob/ob mice (both sexes) to levels of lean mice, this was not due to a change in CFTR expression, which remained diminished. CFTR activation is via a cAMP-dependent pathway, and cellular levels of cAMP are controlled by a balance of cAMP production and degradation (via phosphodiesterases, PDE’s). Inhibition of PDE1, and PDE3 in T84 colonic cells stimulates Cl\textsuperscript-- secretion [39]. Within murine intestine, the presence of PDE1 (calcium/calmodulin-dependent), PDE3 (cGMP inhibited), PDE4 (cAMP-specific), and PDE5 (cGMP-specific) have been noted, and the inhibition of each has resulted in increased jejunal I\textsubscript{sc} [40]. In guinea pig lungs, genistein has been shown to inhibit PDE’s 1-4 [41, 42]. Acute application of genistein to murine jejunum, reduced the effect of milrinone on I\textsubscript{sc}, suggesting that genistein activated Cl\textsuperscript-- secretion by inhibition of a PDE3-dependent pathway (PDE3 can hydrolyze both cAMP and cGMP) [14]. Our data supports those findings in ob/ob female mice, and with chronic application of genistein diet (4-weeks), basal I\textsubscript{sc} is elevated (mimicking that of lean) yet interestingly, this does not appear to be a pathway in ob/ob males.

Here, as with our earlier observations [8] we found no role for the Ca\textsuperscript2+-activated Cl\textsuperscript-- channel, (no effect of DIDS) in ob/ob mice [18]. Secretion from intestinal crypts also involves the activation of clotrimazole-sensitive basolateral K\textsubscript{sc} channels [18, 23, 43]). Our data indicate that the contribution of basolateral (K\textsubscript{sc}) channels towards murine jejunum basal I\textsubscript{sc} is reduced in ob/ob mice. Interestingly, genistein diet rescued this in female ob/ob mice but not in males. We have previously noted sex-dependent mechanisms of action of genistein in ovariecotomized mice [44]. The Na\textsuperscript+/K\textsuperscript+ -ATPase is required for optimal jejunal crypt secretion. Inhibition of the Na\textsuperscript+/K\textsuperscript+ -ATPase with ouabain suggested that the contribution of Na\textsuperscript+/K\textsuperscript+ -ATPase towards basal I\textsubscript{sc} was less in ob/ob mice. Genistein diet rescued the loss of ouabain-sensitive I\textsubscript{sc} in male mice without altering expression of Na\textsuperscript+/K\textsuperscript+ -ATPase. Within the jejunum the Na\textsuperscript+/K\textsuperscript+ -ATPase generates a sodium gradient which drives glucose absorption. Our data is not consistent with Serhan et al. [45], demonstrating that insulin increases glucose uptake with reductions in Na\textsuperscript+/K\textsuperscript+ -ATPase expression, and increased expression of SGLT1 in Caco-2 cells and rat jejunum. The NKCC1 co-transporter is the pathway for Cl\textsuperscript-- entry into jejunal epithelium. Here, we note that bumetanide-sensitive I\textsubscript{sc} is variable in male ob/ob mice (potentially reflecting the reduced total expression of NKCC1 which was reversed by genistein-diet), but was unchanged in females. Interestingly, Ueda-Nishimura et al. [46] have demonstrated that genistein and insulin synergistically stimulate insertion of NKCC1 into renal epithelial cells. Disparities in our data and other studies [46] could be attributed to: differences in cell type (renal versus jejunum, or cells versus intact tissue), the dose of genistein (low micromolar range versus 100 µM) and duration of genistein exposure (4-weeks versus acute application). The importance of sex-dependent differences is an increasing area of focus: greater expression of voltage-dependent K\textsuperscript+ channels in coronary vessels in male swine [47], reduced expression of repolarizing K\textsuperscript+ channel subunits in human female hearts [48], reduced colonic ER\textalpha expression in males [49].

Evidence suggests that intestinal inflammation can modify ionic transport, via changes in expression, function, or modification of the signaling pathways of Na\textsuperscript+/K\textsuperscript+ -ATPase, NKCC1, sodium channels and CFTR [50, 51]. For example, in experimental colitis, reduced colonic I\textsubscript{sc} is due to reduced chloride/bicarbonate secretion [51, 52]. Indeed, TNF-α or IFN-γ downregulate CFTR expression [53, 54]. Given the association of diabetes with inflammation, this fits with our quantified loss of jejunum CFTR expression in ob/ob mice, and loss of NKCC1 expression in male ob/ob mice, and whether or not this is linked to changes in inflammatory cytokines.
remains to be seen. Furthermore, debate continues as to the effect of diabetes on epithelial barrier integrity: Stenman et al. [55] showed that ob/ob mice had no change in gut barrier function, however, other studies suggested that the gut barrier was impaired in ob/ob mice [56].

In conclusion, this study demonstrates that reduced basal I_{sc} in ob/ob jejunum is attributed to a combination of decreases in: total epithelial protein expression of CFTR, contribution of basolateral K_{Ca} channels and Na^{+}/K^{+}-ATPase. We conclude that consuming genistein-diet (600G) for 4-weeks reversed the deficit in basal I_{sc} via the following sex-dependent mechanisms: in females; modification of the PDE3-dependent pathway and increased contribution of basolateral K_{Ca} channels, whereas, in males; an increased contribution of Na^{+}/K^{+}-ATPase, and NKCC1 were noted. While this work contributes significantly towards the current limited understanding of small intestinal function in diabetes, and the complex mechanism(s) of action of genistein thereon, future studies will further examine potential additional cellular mechanisms involved.

Abbreviations

CFTR (cystic fibrosis transmembrane conductance regulatory protein); NKCC1 (Na^{+}/K^{+}/2Cl⁻ cotransporter); I_{sc} (transepithelial short circuit current); IBMX (8-methoxymethyl-3-isobutyl-1-methylxanthine); EHNA (erthyro-9-(2-hydroxy-3-nonyl)adenine); ER/PI3K (estrogen receptor/phosphatidylinositol-3-kinase); PDE (phosphodiesterase enzyme); H & E (hematoxylin and eosin); DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate).

Acknowledgements

The authors would like to thank most sincerely, Dr. Christian Lytle (University California, Riverside) for generous gift of NKCC1 antibody. Shawn Catmull and Robert Dolan were supported by the Midwestern University DO Summer Fellowship Program. Thanks to Ms Meghan Franco for technical assistance. Thanks to APS for supporting Amy Anderson (Basis High School, Phoenix) with a Frontiers in Physiology Research Award. This work was supported by Midwestern University Intramural funds (to L.A.), The Soy Health Research Program (to L.A.), and Diabetes Action and Research Education Foundation (to L.A.).

Disclosure Statement

None.

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