Inhibiting Inducible Nitric Oxide Synthase in Enteric Glia Restores Electrogenic Ion Transport in Mice with Colitis

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Abbreviations

1400W, N-(3-Aminomethyl) benzylacetamidine; BDD, boron-doped diamond; cAMP, cyclic adenosine monophosphate; DCA, deoxycholic acid; DSS, dextran sodium sulfate; EFS, electrical field stimulation; ENS, enteric nervous system; IBD, inflammatory bowel disease; IL, interleukin; I_{SC}, short-circuit current; NO, nitric oxide; NOS, nitric oxide synthase; SEM, standard error of the mean; SMTC, S-Methyl-L-thiocitrulline; TNBS, trinitrobenzene sulfonic acid; TNFα, tumor necrosis factor α; TTX, tetrodotoxin; VIP, vasoactive intestinal peptide.

Key Words: IBD; inflammation; myenteric plexus; glial cells; vasoactive intestinal peptide.

Abstract

Background & Aims: Disturbances in the control of ion transport lead to epithelial barrier dysfunction in patients with colitis. Enteric glia regulate intestinal barrier function and colonic ion transport. However, it is not clear whether enteric glia are involved in the epithelial hypo-responsiveness. We investigated enteric glial regulation of ion transport in mice with trinitrobenzene sulphonic acid- or dextran sodium sulfate-induced colitis and in $Il10^{-/-}$ mice.

Methods: Electrically-evoked ion transport was measured in full-thickness segments of colon from CD1 and $Il10^{-/-}$ mice with or without colitis in Ussing chambers. Nitric oxide (NO) production was assessed using amperometry. Bacterial translocation was investigated in the liver, spleen and blood of mice. **Results:** Electrical stimulation of the colon evoked a tetrodotoxin-sensitive chloride secretion. In mice with colitis, ion transport almost completely disappeared. Inhibiting inducible NO synthase (NOS2), but not neuronal NOS (NOS1), partially restored the evoked secretory response. Blocking glial function with fluoroacetate, which is not a NOS2 inhibitor, also partially restored ion transport. Combined NOS2 inhibition and fluoroacetate administration fully restored secretion. Epithelial responsiveness to vasoactive intestinal peptide was increased after enteric glial function was blocked in mice with colitis. In colons of mice without colitis, NO was produced in the myenteric plexus almost completely via NOS1. NO production was increased in mice with colitis, compared to mice without colitis; a substantial proportion of NOS2 was blocked by fluoroacetate administration. Inhibition of enteric glial function *in vivo* reduced the severity of trinitrobenzene sulphonic acid -induced colitis and associated bacterial translocation.

Conclusions: Increased production of NOS2 in enteric glia contributes to the dysregulation of intestinal ion transport in mice with colitis. Blocking enteric glial function in these mice restores epithelial barrier function and reduces bacterial translocation.

Introduction

Inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are characterized by diarrhea, weight loss, malabsorption of water and nutrients, resulting in debilitating illness[1, 2]. The dysregulation of water and ion transport occurs as a result of altered epithelial function and is directly involved in the symptoms of IBD. However, the mechanisms underlying this dysfunction are largely unresolved and treatment options are limited[3].

Increased NO production has been implicated in intestinal inflammation, although the function of NO has been debated as both pro- and anti-inflammatory[4, 5]. The increase in NO occurs through upregulation of NOS2 in epithelial and immune cells, and in neurons and enteric glial cells of the enteric nervous system (ENS)[6-11]. Enteric glia, long considered to be primarily supportive cells of the ENS, are now known to be actively involved in the maintenance of epithelial barrier function[12]. NOS2-derived NO has been shown to induce epithelial hyporesponsiveness to secretagogues that normally induce apically directed chloride secretion[13, 14]. We have recently described a role for enteric glia in the modulation of nicotinic cholinergic receptor-mediated ion transport[15]. However, whether enteric glia are involved in dysregulating intestinal ion transport during intestinal inflammation is not known.

During intestinal inflammation, enteric glia undergo reactive gliosis[16, 17], respond to and produce inflammatory mediators[16, 18, 19] and are involved in the breakdown of epithelial barrier function[20, 21]. It has been speculated that enteric glia are involved in the regulation of ion transport during experimental colitis, based on the localization and upregulation of NOS2 in

enteric glia during intestinal inflammation[8]. However, a functional role for NOS2 in enteric glia remains to be established.

We tested the hypothesis that enteric glia of the ENS are involved in the dysregulation of ion transport in colitis by directly targeting them using the glial metabolic inhibitor fluoroacetate. Fluoroacetate has been well characterized as a method of reversibly inhibiting glia metabolic function in the central nervous system, where it is preferentially taken up by astrocytes and their function is reversibly inhibited[22-24]. Enteric glia have many similarities to astrocytes[25, 26], and within the ENS, the fluoroacetate metabolite fluorocitrate has been successfully used to demonstrate their role in the regulation of motility[27]. Here we utilize fluoroacetate in conjunction with measurement of intestinal ion transport and NO release from the myenteric plexus to examine the role of enteric glia in the dysregulation of ion transport in the inflamed colon in three mouse models of colitis. We also assessed human colonic biopsies to establish if in the absence of the enteric plexuses we would observe epithelial hyporesponsiveness and if so, if that was reversed by treatment with fluoroacetate.

Methods

Animals. Wild-type male CD1 mice (6-8 weeks; Charles River, Montreal, QC) were used unless otherwise stated. A genetic model of colitis using $Il10^{-/-}$ mice generated on a 129Sv/Ev background was also used, and compared with Sv/Ev wildtype controls. $Il10^{-/-}$ mice develop intestinal inflammation as they age, and therefore mice 16-20 weeks of age were used. Animal protocols were approved by the University of Calgary Animal Care Committee, and were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Animals were housed in polystyrene cages with free access to food and tap water and maintained on a 12h light-dark cycle in a temperature and humidity controlled room. All animals were killed by cervical dislocation under deep isofluorane anesthesia.

Colitis. Colitis was induced using TNBS (0.1mL of 30mg/mL TNBS in 30% ethanol) administered 3 cm intrarectally in animals lightly anesthetized with isofluorane, and DSS (5% w/v) dissolved in drinking water for 5 days followed by 2 days of normal drinking water. TNBS-treated animals were sacrificed 3 days post-treatment and DSS-treated animals were sacrificed 7 days after treatment. The extent of damage induced by intestinal inflammation was assessed in each model using a modified version of an established macroscopic damage score[28, 29]. Briefly, the colonic damage score consisted of the length of damage or length of ulceration (cm), summed with scores for the presence or absence of erythema (0 or 1), diarrhea (0 or 1; defined as loose or watery stool), fecal blood (0 or 1), and adhesions (0, 1 for mild, or 2 for severe). Length of colon (cm) was also recorded.

Chronic TNBS colitis. TNBS colitis was induced in mice as described above and animals were subsequently allowed to recover for 3 weeks. Animals had similar initial weight loss as the acute TNBS-treated mice (Table 1).

Measurement of Electrogenic Ion Transport. Full-thickness distal colonic segments were mounted in Ussing chambers (0.5 cm^2 opening) and held under voltage-clamp conditions in oxygenated (95% O_2, 5% CO_2) Krebs buffer (pH 7.4) that contained (in mM): NaCl (117), KCl (4.8), $CaCl_2$ (2.5), $MgCl_2$ (1.2), $NaHCO_3$ (25), NaH_2PO_4 (1.2) and D-glucose (11). Electrogenic movement of ions across the epithelium was recorded as short-circuit current (I_{SC}, $\mu A/cm^2$), where the change in I_{SC} (ΔI_{SC}) is the peak post-stimulus value subtracted from the stable pre-stimulus baseline. Drugs were added serosally unless otherwise specified and remained in the bath for the duration of the experiment. Electrical field stimulation (EFS; 50V, 10Hz, 3s) was applied in the presence or absence of the following inhibitors: the NOS1 inhibitor S-Methyl-L-thiocitrulline (SMTC; 10μM, Calbiochem, La Jolla, CA), the NOS2 inhibitor N-(3-Aminomethyl) benzylacetamidine (1400W; 10μM, Calbiochem), and the neurotoxin tetrodotoxin (TTX; 500nM, Tocris Bioscience, Ellisville, MO). Additionally, the following agonists were used: substance P (100μM, Sigma-Aldrich, Oakville, ON), and vasoactive intestinal peptide (VIP; 100nM, Calbiochem), the specificity of which was assessed with the VIP antagonist [D-*p*-Cl-Phe[6], Leu[17]]-VIP (300nM, Tocris). At the conclusion of each experiment the adenylate cyclase activator forskolin (10μM, Sigma) was added to the chamber. The involvement of chloride ions was assessed using chloride-free Krebs buffer (pH 7.4) that contained (in mM): D-gluconic acid, sodium salt (117), D-gluconic acid, potassium salt (4.8), $NaHCO_3$ (25), NaH_2PO_4 (1.2), D-gluconic

acid, hemimagnesium salt (1.2), D-gluconic acid, calcium salt (2.5), and D-glucose (11).

Electrochemical Detection of Nitric Oxide. Continuous amperometric monitoring of NO release was conducted on the mucosal surface of full-thickness colon and on myenteric ganglia in myenteric plexus-longitudinal muscle preparations as previously described[15, 30]. Briefly, an NO oxidation current was recorded using a 40μm diameter boron-doped diamond (BDD) microelectrode (described elsewhere[15, 30, 31]). A stainless steel wire served as the counter electrode and a "no leak" Ag|AgCl electrode (EE009, ESA Biosciences Inc., Sunnyvale, CA) was used as the reference electrode. Amperometric measurements were carried out using a BioStat™ multi-mode potentiostat (ESA Biosciences). The BDD microelectrode was reproducibly positioned over ganglia using a micromanipulator (Model 25033, Fine Scientific Tools, North Vancouver, BC). The electrode was held at a detection potential of +1.0 V vs. Ag|AgCl, which was sufficient to oxidize NO at a mass-transfer limited rate. As previously described[15, 30], tissues were continually perfused with Krebs' buffer at a flow rate of 2 mL/min. For experiments investigating NO release from the mucosa, deoxycholic acid (DCA; Sigma; 1mM) was applied for 30 min and NO release measured at 0 and 30min. The BDD microelectrode was placed 200μm above the mucosal tissue surface for 40s and the currents were recorded at +0.85 V and +1.0 V vs. Ag|AgCl. The difference in the current obtained was due to the oxidation of NO and removed interferences from mucosal melatonin and serotonin release. DCA has previously been demonstrated to cause NO release from NOS2 the intestinal epithelium[32, 33]. For experiments investigating NO release from the myenteric plexus, a local superfusion pipette placed within 100μm of the tissue for 20s containing veratridine (10μM; Sigma) was used to

depolarize enteric neurons in the presence or absence of the inhibitors SMTC (10 µM) and 1400W (10µM).

Bacterial Translocation

Examination of bacterial translocation was performed in mice with TNBS colitis or controls, with animals receiving saline or fluoroacetate injections (1mg/kg, IP) every 12h for 3 days. Mice were euthanized by cervical dislocation under deep isofluorane anesthesia and their blood, liver, and spleen tested for translocated bacteria as previously described[13, 34]. Briefly, blood was collected by cardiac puncture and liver and spleen removed using sterile techniques. Fecal samples acted as a positive control. Each tissue and fecal sample was standardized by weight. The samples were homogenized in 2mL of sterile phosphate buffered saline and serial dilutions of aliquots (0.1ml) were plated onto Columbia blood agar to enumerate total aerobic and facultative bacteria and plated onto MacConkey's agar to enumerate aerobic and facultative gram-negative enteric bacilli. The plates were incubated at 37°C in aerobic conditions and the number of colony forming units (CFU) measured at 24h and 48h.

Fluoroacetate Treatment. The glial metabolic inhibitor fluoroacetate (5mM, Sigma) was applied for 120min in Ussing chamber experiments prior to EFS or the addition of agonists and 60min for dissected longitudinal muscle-myenteric plexus preparations in electrochemical detection studies.

Minimum Inhibitory Concentration Test. Bacterial growth in the presence and absence of fluoroacetate was assessed using the minimum inhibitory concentration test, as previously described[35]. *Escherichia coli* 91801 and *Enterobacter cloacae* 10404 bacteria were cultured in LB media for 24 hours. 100 µl of bacteria at a final concentration of $1x10^6$ CFU/mL was then added into individual wells of a 96 well plate. Each well also received 100µl of FA at concentrations ranging from 0.078-200µM. Plates were sealed and incubated for 48 h and subsequently the O.D.$_{600}$ for each well was measured, giving an estimate of bacterial concentration in the sample.

Human Biopsies. Human colonic biopsy samples were acquired from the distal and rectosigmoid colon during colonoscopy or sigmoidoscopy procedures after informed consent from patients with irritable bowel syndrome (n=2), Crohn's disease (n=5) or ulcerative colitis (n=4). Samples were obtained from endoscopically inflamed (but not ulcerated) or uninflamed/normal regions. Adjacent biopsies were assessed by pathologists to verify the presence or absence of inflammation. Biopsies were immediately placed in cold Krebs/Glucose buffer (Krebs + 10mM glucose) and transported to the lab on ice. Samples were treated with Krebs buffer with or without FA (5mM) in an oxygenated chamber (5% CO_2 + 95% O_2) for 120 min at room temperature. Samples were then mounted into Ussing chambers (Physiologic Instruments, San Diego, CA; biopsy specific P2308 sliders) and stimulated with EFS (50V, 10Hz, 5s). These studies were approved by the University of Calgary Conjoint Human Research Ethics Board.

Statistics. Data are presented as mean ± standard error of the mean (SEM) and were compared using one-way ANOVA followed by post-hoc pair-wise comparisons with Tukey's test, unless otherwise stated. *P* <.05 was accepted as a level of statistically significant difference.

Results

The role of NO and enteric glia in the regulation of baseline electrogenic intestinal ion transport

EFS induced a monophasic increase in I_{SC} (42±13 µA/cm^2; n=6; Figure 1A and 1B). This response was abolished in the presence of TTX (0.5±2 µA/cm^2; n=4; P<.01 vs. vehicle), indicating that it is neurally mediated. The I_{SC} response was due to epithelial chloride secretion, as the ΔI_{SC} was abolished in chloride free buffer (0.5±0.5 µA/cm^2; n=4; P<.001 vs. vehicle). Electrogenic ion transport was unaffected by SMTC, 1400W, or both inhibitors in combination (Figure 1B) in untreated animals or in mice treated with saline enemas.

In order to assess the role of enteric glia we used the metabolic inhibitor fluoroacetate[22-24]. Under control conditions, fluoroacetate treatment did not alter the ΔI_{SC} in response to EFS in untreated animals (41±8 µA/cm^2; n=11; P>.05) or animals treated with a saline-enema (36±6; n=6; P>.05). These data suggest that neither enteric glia nor NO play a regulatory role in electrogenic ion transport under physiological conditions.

Ion transport is inhibited during colitis and is restored by inhibiting NOS2

DSS and TNBS treatment induced weight loss (P<.001 vs. respective controls), extensive inflammation and shortening of the colon (Table 1). Similarly, *Il10$^{-/-}$* mice had extensive intestinal inflammation with damage scores that were comparable to the chemical models of colitis (Table 1).

Following the induction of colitis with either TNBS or DSS, the secretory response to EFS was virtually abolished (Figures 1C and 1D). SMTC had no effect on this response, while 1400W partially restored it, and a combination of SMTC and 1400W were not significantly different to 1400W alone (Figure 1C). These data show that in colitis, NO derived from NOS2 is an important inhibitor of electrogenic ion transport.

Inhibiting enteric glial metabolism restores ion transport in colitis

In both TNBS and DSS colitis, fluoroacetate treatment significantly restored the secretory response to EFS and a combination of fluoroacetate and 1400W fully restored the secretory response (Figures 2A and 2B). TTX completely inhibited the restored response following 1400W treatment in TNBS colitis ($2\pm1\mu A/cm^2$; n=4; P<.01 vs. 1400W) and DSS colitis ($2\pm2\mu A/cm^2$; n=4; P<.001 vs. 1400W), and after fluoroacetate treatment in TNBS colitis ($2\pm1 \mu A/cm^2$; n=3; P<.001 vs. TNBS fluoroacetate) and DSS colitis ($1\pm1 \mu A/cm^2$; n=3; P<.05 vs. DSS fluoroacetate). The EFS response following fluoroacetate treatment was absent in chloride free buffer for both models of colitis (TNBS: $2\pm1 \mu A/cm^2$, n=4; P<.01 vs. saline control; DSS: $1\pm1 \mu A/cm^2$, n=4, P<.01 vs. control). These data show that in colitis, enteric glia are involved in the inhibition of electrogenic neurally-mediated chloride secretion. In $Il10^{-/-}$ mice, the ΔI_{SC} following EFS was greatly reduced compared to SvEv wildtype controls. Fluoroacetate treatment had no effect on the ΔI_{SC} in SvEv mice, but partially restored the secretory response in $Il10^{-/-}$ mice (Figure 2C).

Enteric glial dysregulation is reversed following recovery from colitis

Animals that were allowed to recover for 3 weeks following the induction of TNBS colitis had similar responses to EFS compared to controls in the presence and absence of fluoroacetate (Figure 3A; P>.05). The secretory response to forskolin in TNBS-treated mice was also comparable to controls in the presence and absence of fluoroacetate (Figure 3B; P>0.5).

Restoration of the secretory response to neural stimulation in colitis is partially mediated by VIP

In order to identify a neuronal mediator of the ion transport response after fluoroacetate treatment, we first determined that the effect of fluoroacetate was not due to a change in epithelial responsiveness to the cyclic AMP (cAMP)-dependent secretagogue, forskolin, which acts directly on the epithelium. Forskolin elicited a large secretory response in the distal colon under control conditions, following saline enema treatment (Figures 4A and 4B), and in SvEv mice (111 ± 11 $\mu A/cm^2$; n=3). The response to forskolin was unaffected by fluoroacetate in control and saline treated conditions. The magnitude of the forskolin-induced ΔI_{SC} was significantly lower compared to the respective controls in TNBS (Figure 4A), DSS (Figure 4B), and $Il10^{-/-}$ mice (40 ± 7 $\mu A/cm^2$; n=3; P<.001). The response to forskolin was unchanged by fluoroacetate (Figures 4A and 4B) or 1400W or a combination of both (not shown) following TNBS or DSS treatment. These data suggested that the fluoroacetate treatment did not alter the epithelial responsiveness to forskolin.

We next examined substance P and VIP as they are well-known mediators of epithelial chloride secretion[36-38]. Substance P (100µM) elicited a modest increase in ΔI_{SC} (control: 13±4 µA/cm^2, n=7; saline control: 7±3 µA/cm^2, n=4) that was reduced during intestinal inflammation (TNBS: 4±1 µA/cm^2, n=3; DSS: 1±1 µA/cm^2, n=4; P<.05 vs. respective controls). Fluoroacetate treatment did not restore the secretory response to substance P (TNBS fluoroacetate: 1±1 µA/cm^2, n=3; DSS fluoroacetate: 1±1 µA/cm^2, n=4; P>.05 vs. respective inflamed condition).

VIP (100 nM) elicited a robust increase in ΔI_{SC} under control and saline control conditions that was unchanged by fluoroacetate treatment (Figures 4C and 4D). The selective VIP receptor antagonist [D-p-Cl-Phe^6, Leu^{17}]-VIP (300nM) completely blocked the response to VIP under control (0±2 µA/cm^2, n=3) and saline control (1±1 µA/cm^2, n=3) conditions with or without fluoroacetate treatment (control fluoroacetate: -2±1 µA/cm^2, n=3; saline control fluoroacetate: 0±0 µA/cm^2, n=3; P>.05 vs. respective control). The response to VIP was decreased in TNBS (Figure 4C, P<.001 vs. saline control) and DSS colitis (Figure 4D, P<.001 vs. control). Fluoroacetate treatment partially restored the VIP response in both TNBS (Figure 4C) and DSS (Figure 4D) treated mice. These restored responses were blocked by [D-p-Cl-Phe^6, Leu^{17}]-VIP (TNBS fluoroacetate: 2±1 µA/cm^2, n=3; DSS fluoroacetate: 1±1 µA/cm^2, n=3; P>.05 vs. respective control).

TTX did not alter the ΔI_{SC} response to VIP in saline enema control (Figure 4E) and untreated control conditions (Figure 4F). This response was also unaffected by fluoroacetate. In colitis, TTX did not alter the reduced ΔI_{SC} in response to VIP (data not shown). However, following

fluoroacetate treatment, TTX now blocked the VIP-induced ΔI_{SC} in both TNBS colitis (Figure 4E) and DSS colitis (Figure 4F). These data suggest that VIP is a mediator of the neurally evoked ion transport response that occurs after fluoroacetate treatment in colitis.

Nitric oxide is released from the myenteric plexus following neuronal activation

Having determined that NO from NOS2 and enteric glia is the inhibitory mediator of secretion, we wished to determine whether NO release occurs in the myenteric plexus. Whilst the traditional view of enteric neural regulation of secretion considers that the submucosal plexus is responsible for secretion and the myenteric plexus for motility, our work and that of others suggests that both plexuses together coordinate ion transport[8, 15, 39-41]. Given the extreme technical difficulty in dissecting the isolated submucosal plexus, we investigated NO signaling within the myenteric plexus. We measured NO release following the application of veratridine, which excites enteric neurons[42]. Under physiological conditions, veratridine caused an increase in NO release that was insensitive to fluoroacetate (Figure 5A). This response was largely derived from NOS1 as it was almost completely blocked by treatment with SMTC (Figure 5B). There was a small NOS2 component that was sensitive to 1400W. SMTC and 1400W together completely blocked the NO release (Figure 5B). In TNBS and DSS colitis, veratridine caused an increase in NO release that was larger than that observed in controls and was only partially blocked by SMTC, significantly reduced by 1400W, and was completely inhibited by SMTC and 1400W together (Figures 5C and 5D).

Inhibiting enteric glial metabolism blocks NO release from the myenteric plexus during intestinal inflammation

We next assessed if inhibiting glial metabolism would decrease the release of NO in colitis. In both TNBS- and DSS-induced colitis, tissues treated with fluoroacetate had reduced NO release from the myenteric plexus following veratridine stimulation compared to untreated TNBS tissue (P<.001). This was completely inhibited by SMTC or in combination with 1400W (Figures 5E and 5F), but unchanged by 1400W alone. These data suggest that fluoroacetate inhibits the ability of enteric glia to be stimulated by enteric nerves to produce NO from NOS2.

Fluoroacetate is not a nitric oxide synthase 2 inhibitor

We examined the ability of fluoroacetate to inhibit NO production using DCA, which stimulates NO production from NOS2 in the intestinal mucosa. In the absence of stimulation, the mucosa had a low basal production of NO. DCA treatment increased the production of NO, which was completely blocked by 1400W (Figure 6). The effect of DCA remained unchanged by fluoroacetate treatment (Figure 6). These data suggest that fluoroacetate is not a NOS2 inhibitor and reaffirms its preferential inhibitory activity on glia.

Bacterial translocation following intestinal inflammation was decreased by *in vivo* fluoroacetate treatment

We assessed the effect of fluoroacetate treatment *in vivo* on damage score and bacterial translocation. In animals without colitis, no damage score was recorded. The average damage score of TNBS treated animals was 10.1±1 (n=9) and fluoroacetate treatment reduced the

average damage score to 6.3±1 (n=7; P<.05 vs. TNBS), with the major difference in damage score being an absence of ulcerations in fluoroacetate-treated animals. In animals without colitis, no bacterial translocation was detected (Table 2). However, in TNBS treated animals, hemolytic bacteria were found in 22% of blood samples, 56% of liver samples, and 78% of spleen samples. Gram negative bacteria were found in 11% of blood samples, 67% of liver samples, and 33% of spleen samples. In animals with TNBS colitis treated with fluoroacetate, hemolytic bacteria were found in 0% of blood samples, 14% of liver samples, and 14% of spleen samples (P<.05 vs. TNBS spleen; Fisher's exact test). Gram negative bacteria were found in 0% of blood samples, 14% of liver samples, and 14% of spleen samples.

Finally, we investigated whether fluoroacetate was acting as an antibacterial agent; instead of preventing bacterial translocation it could be killing bacteria present in the gut, blood, and organs. Over a range of concentrations, fluoroacetate (0.078-200 µM) did not alter the growth of *Escherichia coli* 91801 and *Enterobacter cloacae* 10404 bacteria in culture.

Colonic biopsies from IBD patients have normal ion transport

We found no difference in the ion transport response to EFS between biopsies from the inflamed (17.0 ± 4.5 µA/cm^2; +FA 18.9 ± 4.7 µA/cm^2; n=3) and uninflamed (19.0 ± 3.4 µA/cm^2; +FA 14.8 ± 2.4 µA/cm^2; n=11) regions of the colon in the presence or absence of fluoroacetate.

Discussion

The aim of this study was to investigate the role of NO and enteric glia on the regulation of epithelial ion transport during intestinal inflammation. Using chemical and genetic mouse models of colitis and the glial-specific metabolic inhibitor fluoroacetate, we have demonstrated that enteric glia inhibit electrogenic epithelial ion transport through NO release from NOS2 during colitis. This occurs independently of the neuronal NOS isoform NOS1. The functional significance of these pathways was shown by inhibiting enteric glial function *in vivo*, substantially reducing inflammation and bacterial translocation in colitis.

Our findings in colitis are in contrast to the normal physiological regulation of electrogenic epithelial ion transport in the colon. The increase in chloride secretion in control mice appears to be mediated solely by enteric nerves, since neither glial metabolic inhibition nor the inhibition of NO release have an effect. This finding is supported by previous work in the ENS using the fluoroacetate metabolite fluorocitrate, which did not inhibit ion transport *in vivo* under physiological conditions[27]. These results are interesting given our previous findings that nicotinic cholinergic signaling in myenteric ganglia mobilizes NO from NOS2 in enteric glia, which modulates epithelial ion transport under physiological conditions[15]. In contrast to nicotinic cholinergic stimulation, EFS releases many excitatory and inhibitory neurotransmitters from neurons within the ENS, including acetylcholine, substance P and other tachykinins, VIP, adenosine triphosphate, serotonin, and NO[43]. Given that nicotinic cholinergic activation represents only one component of this complex system, it is perhaps not surprising that there are differences when nicotinic stimulation is compared to EFS.

During intestinal inflammation, the intestinal epithelium and neural secretomotor pathways are hyporesponsive to secretory stimuli, including the cAMP secretagogue IBMX, acetylcholine, and neural stimulation, persisting even after active inflammation has resolved[8, 44-49]. The mechanisms mediating epithelial ion transport hyporesponsiveness are not well understood[3]. The ENS has been implicated, since removal of the submucosal plexus reverses the hyporesponsiveness of the epithelium[3]. Nitric oxide has also been implicated; in a mouse model of colitis, inhibiting NOS2 activation partially restored physiological ion transport following a neural-specific stimulus[48]. The upregulation of NOS2 during intestinal inflammation has been well established[7-9]. We confirmed that neural stimulation during intestinal inflammation resulted in greater NO release, due to an increase in NOS2 activity in the myenteric plexus. Additionally, we further investigated the role of NO and confirmed the findings of MacNaughton *et al.*[48] demonstrating that it was specifically NOS2 inhibition which restored ion transport, while NOS1 inhibition had no effect. Enteric glia are known to be a major source of NOS2 within the ENS, and a role for enteric glia in the regulation of epithelial ion transport during intestinal inflammation has been speculated[8]. Using fluoroacetate to directly investigate the role of enteric glia, we substantially reversed the inhibition of electrogenic ion transport following intestinal inflammation, suggesting that enteric glia play an important role in the inhibition of epithelial ion transport during colitis. Previous work in the ENS has observed that fluorocitrate appears solely to inhibit energy dependent processes in enteric glia[27]. In our study, fluoroacetate prevented NO release specifically from NOS2 without altering NOS1-mediated NO release or inhibiting NOS2 itself, suggesting that fluoroacetate directly targets enteric glial metabolism.

An interesting observation was the additive effect of the inhibition of NOS2 and metabolic inhibition of enteric glia in TNBS colitis. In contrast, the electrochemical data suggested that enteric glial NO release from the myenteric plexus is completely inhibited following either the inhibition of NOS2 or enteric glial metabolism. There is a difference between these preparations that helps explain these data. Nitric oxide sensing was done on longitudinal muscle-myenteric plexus preparations, while the ion transport experiments were conducted in full-thickness tissue. Therefore there may be incomplete inhibition in the full-thickness tissue due to penetrance issues. Alternatively, other cellular components or factors regulate the release of NO within the gut wall. The intestinal epithelium itself produces NO during intestinal inflammation[10, 11]. Additionally, infiltrating immune cells could produce NO and modulate the system, although this has not been explored. Finally, other glial populations are present in the gut such as mucosal glia, which help maintain the epithelial barrier and play a role in antigen presentation[12, 16, 20]. However, we do not yet have an adequate way to isolate glial subtypes.

To preclude model-specific effects of our observations, three different models of murine colitis were tested. The chemical models, TNBS and DSS, are acute models of colitis characterized by extensive epithelial damage, although with significant differences in the manifestation of this damage. The $Il10^{-/-}$ model represents a chronic inflammatory model that develops postnatally and persists over time. All three models have impaired electrogenic ion transport that is reversed by inhibiting glial metabolism. The commonality of enteric glial regulation of intestinal ion transport via NO across multiple models provides good evidence for a common mechanism that may be relevant in IBD.

Chloride secretion is regulated at the level of the epithelium primarily by cAMP, but impaired cAMP-mediated secretion during colitis was not altered by fluoroacetate. Similarly, substance P had an impaired secretory response during intestinal inflammation that was unaltered by fluoroacetate. These observations suggest that enteric glia do not directly mediate epithelial responsiveness to secretagogues. However, VIP, another modulator of ion transport, had an impaired secretory response during intestinal inflammation that was partially reversed by fluoroacetate, and was sensitive to TTX, suggesting that VIP may modulate electrogenic ion transport with actions on or through both neurons and enteric glia. VIP has been identified in the majority of neurons in the submucosal plexus of the middle and distal mouse colon[50] and can act as a non-adrenergic non-cholinergic inhibitory neurotransmitter within the ENS, similar to NO[38, 51]. Indeed, VIP colocalizes with NOS1 in the myenteric plexus[37, 52, 53]. Nitric oxide can stimulate VIP release as demonstrated through the application of NO donors, and NOS inhibitors can block VIP release[54, 55]. VIP release could be altered by NOS2 inhibition or the metabolic inhibition of enteric glia during colitis, and therefore when enteric glia are metabolically inhibited, enteric neurons are uninhibited, leading to the restoration of the electrically-evoked secretory response during colitis.

Interestingly, this mechanism of enteric glial dysfunction does not seem to persist following the resolution of intestinal inflammation. In TNBS treated mice, normal secretory function was restored following a three-week post-inflammation period, and was unaltered by fluoroacetate, suggesting that the process in which enteric glia dysregulate ion transport is pathological.

We used human biopsy samples in Ussing chambers to describe ion transport in the distal and/or rectosigmoid colon of patients with IBD, comparing inflamed and uninflamed regions from these patients. We found no difference in the ion transport response to EFS between the inflamed and uninflamed regions of the colon in the presence or absence of glial metabolic poison fluoroacetate. Responses to electrical field stimulation in these preparations are likely due to direct axonal activation and because the neurons of the ENS are mostly absent, these results are consistent with our findings in the mouse. The lack of difference in EFS response in inflamed vs. uninflamed tissues suggests that glia-derived NO is acting on neuronal cell bodies in the ENS and not at the level of the epithelium. At this point additional human tissue experiments in full-wall thickness preparations is required to confirm the results of our findings in mice.

The functional consequences of our findings are important as we found that fluoroacetate treatment decreased or prevented bacterial translocation and reduced colitis, without any antibacterial effects on the growth of common strains of bacteria found in normal gut flora. Fluoroacetate restored chloride secretion in colitis, which is a known mechanism of host defense against bacteria and other pathogens crossing into the gut tissue from the lumen[56]. The absence of appropriate chloride secretion during intestinal inflammation may allow bacterial translocation, precipitating or exacerbating inflammation. The inhibition of enteric glia and their production of NO may reverse this effect and permit chloride secretion to occur, thereby preventing this harmful process.

In conclusion, we have demonstrated a novel role for enteric glia located in the ENS in the dysregulation of intestinal ion transport during colitis. Enteric glial-derived factors, including NO, may have detrimental functional consequences on the integrity of the epithelial barrier, and, as a result, on the severity of colitis. Additionally, these findings provide a deeper understanding of the communication between neurons and enteric glia in the dysregulation of homeostasis during intestinal inflammation, and suggest that enteric glia could be a novel therapeutic target in the treatment of IBD.

FIGURE LEGENDS

Figure 1. A. Recordings demonstrating short-circuit current (I_{SC}) response to EFS and forskolin (FSK) in full-wall thickness colon (I.), I_{SC} in the presence of TTX (II.) and in chloride-free buffer (III.) from control mice. **B.** EFS responses in the presence of NOS1 (SMTC), NOS2 (1400W) and NOS1 and NOS2 inhibitors (n=6/group). **C.** EFS responses in saline control and TNBS colitis in the presence and absence of NOS1 and NOS2 inhibitors (n=4/group). **D.** EFS responses in control and DSS colitis in the presence and absence of NOS1 and NOS2 inhibitors (n=4-10/group). *P<.05; **P<.01; ***P<.001 compared to non-inflamed control; $^{#}$P<.05; compared to inflamed control.

Figure 2. EFS responses in control (**A.**, **B.**), TNBS colitis (**A.**) and DSS colitis (**B.**) in the presence and absence of fluoroacetate (FA) and FA in combination with the NOS2 inhibitor 1400W during TNBS colitis (n=6-10/group). **C.** EFS responses in $Il10^{-/-}$ mice and their wildtype controls (SvEv, n=3) in the presence and absence of FA. *P<.05; **P<.01; ***P<.001 compared to non-inflamed control or as specified; $^{#}$P<.05; $^{###}$P<.001 compared to inflamed control.

Figure 3. A. I_{SC} responses to EFS in the presence and absence of fluoroacetate (FA) in saline control and 3 weeks after TNBS treatment (n=5-9). **B.** I_{SC} responses to EFS in the presence and absence of forskolin (FSK) in saline control and 3 weeks post TNBS treatment (n=4-7).

Figure 4. A. & B. I_{SC} responses to forskolin (FSK) in the presence and absence of fluoroacetate (FA) in control (**A.**, **B.**), TNBS colitis (**A.**) and DSS colitis (**B.**). Note that the FSK response was greatly reduced by colitis and not altered by FA (n=6-18/group). **C. & D.** I_{SC} responses to VIP in the presence and absence of FA in TNBS colitis (**C.**) and DSS colitis (**D.**). Note that the

responses to VIP are significantly greater in the presence of FA (n=5-9/group). **E. & F.** I_{SC}

responses to VIP in the presence of TTX and FA + TTX in TNBS colitis (**E.**) and DSS colitis (**F.**).

Note that TTX blocked the ability of FA to restore the VIP responses (n=3/group). *P<.05;

**P<.01; ***P<.001 compared to non-inflamed control; #P<0.05 compared to inflamed control.

Figure 5. A. Recording of nitric oxide (NO) production from the myenteric plexus following

veratridine stimulation in the presence and absence of fluoroacetate (FA) under control

conditions. **B.** NO production from the myenteric plexus from control animals in response to

veratridine in the presence and absence of NOS1 (SMTC), NOS2 (1400W) and NOS1 and NOS2

inhibitors (n=5-6/group). **C. & D.** NO production from the myenteric plexus in response to

veratridine from animals with TNBS colitis (**C.**) and DSS colitis (**D.**) in the presence and absence

of NOS1 (SMTC), NOS2 (1400W) and NOS1 and NOS2 inhibitors (n=3-6/group). **E. & F.** NO

production from the myenteric plexus in response to veratridine from animals with TNBS colitis

(**E.**) and DSS colitis (**F.**) treated with FA in the presence and absence of NOS1 (SMTC), NOS2

(1400W) and NOS1 and NOS2 inhibitors (n=3-6/group). ***P<.001 compared to veratridine;

#P<.05; ###P<.001 compared to veratridine + 1400W.

Figure 6. DCA stimulated NO production from the mucosa in the presence of the NOS2

inhibitor 1400W and fluoroacetate (FA). Note that FA has no effect on DCA-induced NO
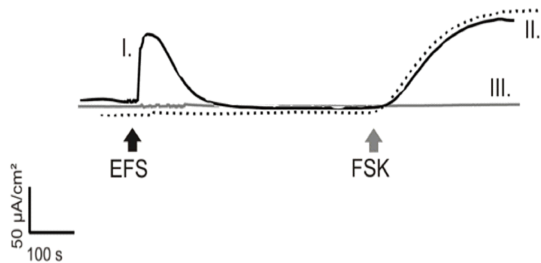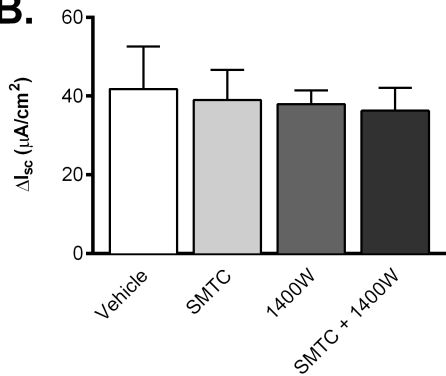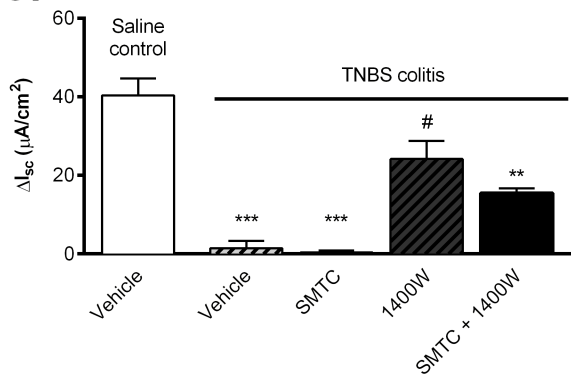
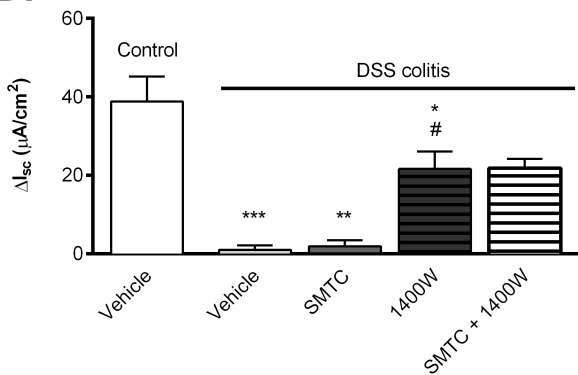production (n=4-10/group). ***P<.001.

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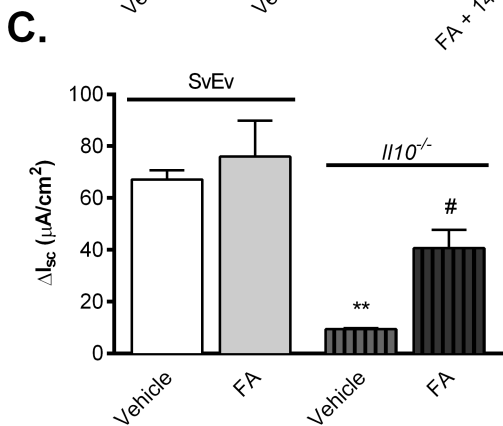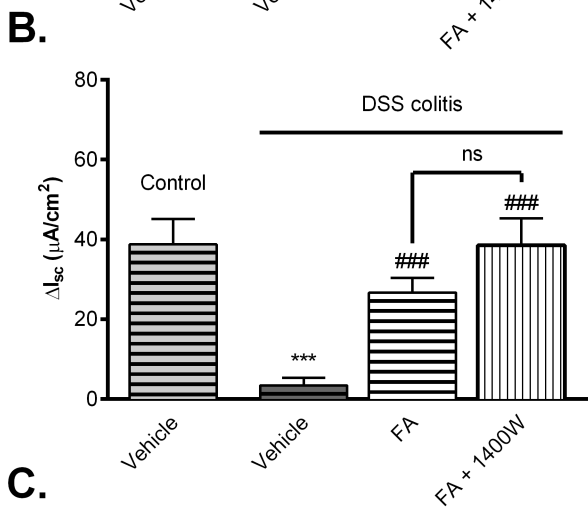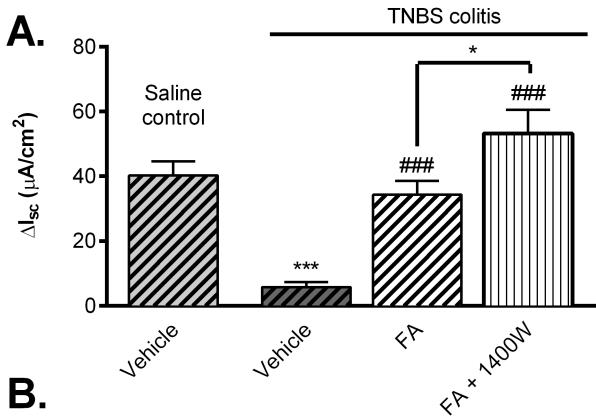
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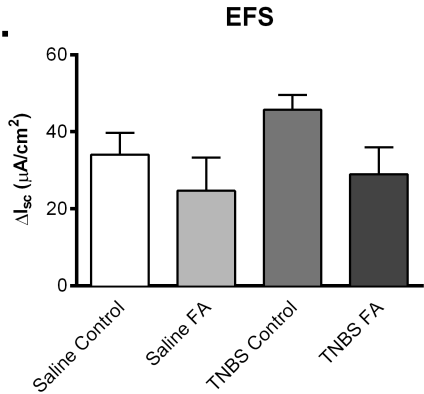
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A.

TNBS colitis

Saline control

ΔI_{sc} ($\mu A/cm^2$)

*

###

Vehicle   Vehicle   FA   FA + 1400W

***   ###

B.

DSS colitis

Control

ΔI_{sc} ($\mu A/cm^2$)

ns

###

Vehicle   Vehicle   FA   FA + 1400W

***

C.

SvEv   *Il10*$^{-/-}$

ΔI_{sc} ($\mu A/cm^2$)

#

Vehicle   FA   Vehicle   FA

**