(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 March 2009 (19.03.2009)

PCT

(10) International Publication Number
WO 2009/035497 A2

(51) International Patent Classification:
A61K 38/16 (2006.01) *A61P 1/00* (2006.01)
A61P 29/00 (2006.01)

(21) International Application Number: PCT/US2008/009496

(22) International Filing Date: 8 August 2008 (08.08.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/963,969 8 August 2007 (08.08.2007) US

(71) Applicant and
(72) Inventor: SAVIDGE, Tor, C. [GB/US]; 2150 Brittany Colony Drive, League City, TX 77573 (US).

(74) Agent: ADLER, Benjamin, Aaron; Adler & Associates, 8011 Candle Ln., Houston, TX 77071 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

WO 2009/035497 A2

(54) **Title:** DISEASE RELATED CYSTEINE MODIFICATIONS AND USES THEREOF

(57) **Abstract:** The present invention discloses a role for s-nitrosoglutathione in the loss of epithelial barrer integrity. The present invention is further directed to examining the therapeutic value s-nitrosoglutathione and/or a chemical entity that modifies cysteine thiol groups in regulation of intestinal mucosal integrity, function, inflammation, and pain by establishing animal models. This use of s-nitrosoglutathione and/or a chemical entity that modifies cysteine thiol groups provides a novel strategy for therapeutic intervention of pathologies associated with inflammatory barrier disorder such as inflammatory bowel disease, functional bowel disease, and infectious diarrheal disease.

5 # DISEASE RELATED CYSTEINE MODIFICATIONS AND USES THEREOF

10 ## BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the fields of microbiology, toxicology, immunology, pharmacology and mucosal inflammatory permeability disorders. More specifically, the present invention 15 discloses the regulation of tissue permeability and dysfunction by cysteine modification and uses thereof.

Description of the Related Art

Permeability barriers that are essential for normal function of the gut and brain exist across the mucosal epithelia and the cerebral endothelia that form the blood-brain barrier. These barriers regulate 20 movement of solutes and macromolecules across paracellular pathways to maintain tissue homeostasis. In both cases, the barriers result from membrane specializations involving the arrangement of complex intercellular adherens and tight-junctions and polar membranes coupled with low rates of pinocytosis.

The intestinal epithelial cells and blood-brain barrier-associated endothelia that form these tight-junctions share specific similarities such as high transcellular resistances and increased levels of P-25 glycoprotein, aquaporins, γ-glutamyl transpeptidase activity and glucose transport. Rapid solvent and nutrient transport are facilitated in both cell types by the high degree of polarity and electrical resistances that are present across the tight-junctions. Barrier disruption complicates both gastrointestinal and neurological diseases and is associated with inflammatory disorders.

Within the central nervous system (CNS), evidence indicates that the blood-brain barrier is 30 formed and maintained via interactions between astrocytes and cerebral endothelia, and evidence is building that astroglial-derived soluble mediators induce blood-brain barrier function. In contrast, there is little or no information available about the role of interactions between glia and epithelial cells in inducing the mucosal barrier. In the gastrointestinal tract, enteric glial cells are abundant and provide regulatory signals for the development and function of neurons in a similar manner to CNS astrocytes. Within the intestinal mucosa, 35 enteric glial cell bodies are in close proximity (<1 μm) of the epithelial border and end-feet processes often appear to directly contact the epithelial basement membrane and blood capillaries in the lamina propria.

Conditional ablation of enteric glia in transgenic mice expressing either the herpes simplex virus thymidine kinase gene (HSVtk) or the influenza virus haemagglutinin receptor from the astroglial specific promoter for glial fibrillary acid protein (GFAP) results in fulminant intestinal inflammation. 40 Intestinal pathology in GFAP-HSVtk transgenic mice treated with the antiviral drug ganciclovir is preceded by

the loss of peripheral GFAP-expressing enteric glia in the distal small intestine and an apparent disruption of the intestinal epithelial monolayer. This observation suggested that mucosal barrier function might be adversely affected by enteric glial cell dysfunction and that interactions between enteric glia and mucosal epithelia may have implications in intestinal inflammatory permeability syndromes where the enteric glial cell network is disrupted.

The prior art is deficient in the mechanism responsible in for maintaining permeability barrier in the intestine. Specifically, the prior art lacks knowledge of the regulation of tissue permeability and dysfunction by cysteine modification and uses thereof. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a method of treating intestinal inflammation and dysfunction in an individual. Such a method comprises administering a pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that post-translationally modifies cysteine thiol moieties in an individual, thereby treating the tissue dysfunction, permeability defect and intestinal inflammation in the individual.

In another embodiment of the present invention, there is provided a method of regulating permeability of mucosal epithelia and the blood brain-barrier. This method comprises contacting an epithelial cell of the mucosal epithelia with a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups. This contact also induces expression of one or more than one epithelial tight-junction associated proteins, transnitrosylation of one or more than one epithelial tight-junction associated proteins, transnitrosylation of toxin released by toxigenic bacteria, inhibition of binding of pathogenic bacteria to mucosal epithelial cells or a combination thereof, thereby regulating the permeability of the mucosal epithelia.

In yet another embodiment of the present invention, there is a method of treating Crohn's disease in an individual. Such a method comprises administering pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual. Such an administration restores the intestinal mucosal barrier function, attenuates inflammation of the colon or a combination thereof, thereby treating the Crohn's disease in the individual.

In yet another embodiment of the present invention, there is provided a method of treating functional bowel disorders in an individual. Such a method comprises administering pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual. Such an administration inhibits associated visceral pain of the colon thereby treating the irritable/functional bowel disease and gastroparesis in the individual.

In yet another embodiment of the present invention, there is provided a method of treating *Clostridium difficile* toxin-induced colitis in an individual. Such a method comprises administering pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual. Moreover, the disease attenuation is further enhanced by treatment with phytic acid/inositol phosphate supplementation Such an administration facilitates inactivation

of the toxin, facilitates restoration the intestinal mucosal barrier function, facilitates attenuation of tissue inflammation or a combination thereof, thereby treating the colitis in the individual.

In yet another embodiment of the present invention, there is provided a method of treating Enterohemorrhagic *Escherichia coli* (EHEC), Shigella flexneri, Enteropathogenic *Escherichia coli* (EPEC), Enterotoxigenic *Escherichia coli* (ETEC), and Enteroaggregative *Escherichia coli* (EAEC)-induced intestinal disease in an individual. Such a method comprises administering a pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual. Such an administration reduces bacterial binding to the epithelium, helps restore the intestinal mucosal barrier function, helps attenuate tissue inflammation or a combination thereof, thereby treating the infective colitis in the individual.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D show that intestinal mucosal barrier function in vivo was regulated by enteric glia. **Figure 1A** shows mice scanned after receiving 0.1 ml PeakFlow™ Infrared flow cytometry reference beads (770 nm emission, 2% solid) using a Li-Cor Odyssey infrared scanner. After 10 min (left) and 180 min (right) the probe (green) was located in the stomach and the jejunal ligature of Tritz, respectively. **Figure 1B** shows serum concentrations (ng/ml) of FITC-dextran (black fill) and fluorescein-5,-6-sulfonic acid (light fill) in nontransgenic (Ntg) and GFAP-HSVtk transgenic (Tg) with and without GCV treatment for 7 d (data are means ± SEM of 4 animals per group; *, $p<0.01$, t-test after passing normality test). **Figure 1C** shows GFAP-HSVtk transgenic (Tg) *gfap* gene expression in the ileum relative to nontransgenic mice (Ntg) after 7 and 14 days of GCV treatment (data are means ± SEM of 4 animals per group; *, $p<0.05$, t-test after passing normality test). **Figure 1D** shows proinflammatory cytokine mRNA abundance and myeloperoxidase activity (MPO) for nontransgenic (Ntg) and GFAP-HSVtk transgenic (Tg) mice receiving GCV for 7 or 14 days respectively. Expression is shown as a fold-increase relative to Ntg 7d controls. Data are means ± SEM of 4 animals per group (*, $p<0.001$, ANOVA).

Figures 2A-2D show that CNS-astrocyte markers were expressed by enteric glia. **Figure 2A** shows that mucosal enteric glial cell processes expressed GFAP and were closely positioned to the murine intestinal epithelium (Mag. X 200). **Figure 2B** shows GFAP expression (green) and DAPI (blue)-labelled nuclei in primary murine enteric glial cell cultures (Mag. X 200) by fluorescent staining. **Figure 2C** shows Li-Cor Odyssey immunoblot of transformed rat enteric glial cells (rEGC) and primary rat enteric glia (pEGC) protein lysates doubled-labeled for p75 (red) and GAPDH (green). **Figure 2D** shows that surface p75 was abundantly expressed in primary rat enteric glia (pEGC) but not in 3T3 fibroblasts by flow cytometry. **Figure 2E** shows the intensity of gene expression for characteristic glial cell markers (vimentin, GFAP, S100β and glutamine synthetase) and potential regulators of blood-brain barrier (TGFβ1-3, GDNF, bFGF, adrenomedullin and endothelin-I) in rEGC and C6 astrocytes using Affymetrix Rat Genome 230 2.0 Gene Chip analysis. Other potential regulators of blood-brain barrier function, notably somatostatin, angiotensin II, taurine, atrial natriuretic factor (ANF), VIP, and melatonin were not expressed in either cell line.

Figures 3A-3D show that epithelial barrier function in vitro was promoted by enteric glia. **Figure 3A** show transepithelial resistances (TER, Ω cm^{-2}) of Caco-2 monolayers grown in co-culture with

primary and transformed enteric glial cell lines from rat (rEGC) or mouse (mEGC), C6 astrocytes and 3T3 fibroblasts (n=3, means ± SEM; *, $p<0.05$ earliest significant time-point relative to Caco-2 cells alone; ANOVA). **Figure 3B** shows Caco-2 monolayers apically pulse-labeled with the permeability markers FITC-dextran (4.4 kD) and fluorescein sulfonic acid (478 Da). Media collected from the basolateral chamber after 4 hrs demonstrated a significant decrease in paracellular movement of fluorescent markers following co-culture with rEGC (n=3, means ± SEM; p=0.02 and p=0.003 for FITC-dextran and Fl-sulfonic acid, respectively; paired t-test after demonstrating normality using the Kolmogorov-Smirnow test). **Figure 3C** shows a Li-Cor Odyssey immunoblot of ZO-1 (red) and e-cadherin (green) protein expression, extracted from Caco-2 cells alone or in co-culture with rEGC for 24 hours. Graph comprising the tight-junction protein expression in soluble and insoluble Caco-2/rEGC co-culture fractions demonstrated that ZO-1 and occludin were significantly up regulated after 24 hours when compared to Caco-2 cells alone (n=3, means ± SEM; *, $p< 0.05$ using t-test after demonstrating normality using the Kolmogorov-Smirnow test). **Figure 3D** show TRITC phalloidin-labelling of MDCK cells expressing dominant-negative rhoA in the presence and absence of rEGC (Mag x200).

Figure 4A-4D show the characterisation of enteric glial-derived barrier-inducing factor (BIF). **Figure 4A** shows transepithelial resistances (TER, Ω cm^{-2}) of Caco-2 monolayers grown in co-culture with rat enteric glia (rEGC) and 3T3 fibroblasts (left) or exposed to rEGC serum-free conditioned media (CM) for 24 hrs (right) (*, $p<0.05$ as compared with Caco-2 cells alone; #, $p<0.05$ as compared with Caco-2 cells co-cultured with 3T3 fibroblasts; n=3, ANOVA). TER of Caco-2 monolayers were significantly increased following treatment with <1kDa, but not with >1kDa ultrafiltrate of rEGC CM (p=0.0014; n=4, t-test after demonstrating normality using the Kolmogorov-Smirnow test). **Figure 4B** shows that transepithelial resistances (TER, Ω cm^{-2}) of Caco-2 monolayers were significantly increased following application of the <1 kDa EGC barrier-inducing fraction (*BIF*) to the basolateral, but not apical, membrane (n=3, means ± SEM; *, p= 0.0036; t-test after demonstrating normality using the Kolmogorov-Smirnow test). **Figure 4C** shows a histogram demonstrating TER-inducing activity following size exclusion chromatography on HR10/30 matrix. Arrow indicates maximum activity in a 300-to-500 Da fraction. **Figure 4D** is tandem mass spectrometric fragmentation spectra that demonstrated GSH and GSNO species (arrows) in HR10/30 active fraction.

Figures 5A-5F show that mucosal barrier function in vitro and in vivo was promoted by GSNO. **Figure 5A** shows that GSNO promoted a significant increase in Caco-2 transepithelial resistance (TER) at concentrations below 150 μM using a dose-response curve. This TER was reversed at concentrations >150μM (n-=3, means ± SEM; *, $p<0.05$ relative to control; ANOVA). **Figure 5B** shows that at a 50 μM concentration, oxidised and reduced forms of GSH demonstrated no significant effect on TER when compared to GSNO (n=3; *, $p<0.01$ relative to control; ANOVA). **Figure 5C** shows that Caco-2 TER was significantly increased by 1kDa fraction of rat EGC conditioned media (rEGC 1kDa), whereas this effect was significantly attenuated when rEGC 1kDA was pretreated with 20 U/ml carboxypeptidase Y, 1 mM glutathione-dependent formaldehyde dehydrogenase (NAD^+, $NADP^+$), 1mM dithiothreitol for 2 hr at 37°C or incubated with L-NAME (100 μM) (n=3; *, $p<0.05$ as compared with Caco-2 cells alone; # $p<0.05$ as compared to Caco-2 cells co-cultured with rEGC 1kDa; ANOVA). **Figure 5D** shows that serum concentrations (ng/ml) of fluorescein-5,-6-sulfonic acid were significantly elevated in GFAP-HSVtk transgenic (Tg) mice given gancilovir for 7 days.

This elevation was completely blocked by GSNO-treatment (n=5, means ± SEM; *, p<0.001 as compared to NTg + Veh; ANOVA). **Figure 5E** shows that GCV (100mg/kg/day) for 11 days had no detectable effect on ileum of non-transgenic (NTg) mice but caused a severe inflammation in GFAP HSVtk transgenic (Tg) mice that was markedly attenuated by simultaneous treatment with GSNO (10mg/kg/day). **Figure 5F** shows that by day 11 GFAP-HSVtk transgenic mice receiving GCV developed fulminant terminal jejuno-ileitis requiring sacrifice based on the probability of survival curves. This effect was delayed by GSNO treatment (n=6 animals per group).

Figures 6A-6C show that mucosal barrier function in human intestine was enhanced by GSNO. **Figure 6A** shows ZO-1 immunolabeling (green) in Caco-2 intestinal epithelial cells (red nuclear counterstain with Syto60; Mag x200). **Figure 6B** shows that relative ZO-1 mRNA and protein expression were increased following GSNO (10 μM) treatment of Caco2 cells for 24 hours (n=3; *, p = 0.018 and 0.035 for mRNA and protein respectively; t-test after demonstrating normality using the Kolmogorov-Smirnow test). **Figure 6C** shows the effects of GSNO on human colonic permeability. Histologically normal colonic biopsies from Crohn's disease (CD-N) or control (Cont) patients without inflammatory bowel disease were maintained in Ussing chambers in the presence of GSNO (100 μM) or vehicle applied to the basolateral chamber for 2 hours. Percentage (%) increases in permeability to FITC-Inulin (4 kDa) applied to the apical chamber are shown relative to starting values (t=0). GSNO significantly attenuated permeability in Crohn's disease but not in control biopsies. Data are means ± SEM of 3 biopsies per group (*, p=0.012).

Figures 7A-7C show that transnitrosylation of purified toxin A with GSNO inhibited the toxicity. **Figure 7A** shows fluid secretion measurements in ileal loops treated with *Clostridium difficile* toxin A, with GSNO (100 uM) and with vehicle control (p<0.05, * and #, significantly different to PBS/Veh and TxA/GSNO, respectively). **Figure 7B** shows real-time quantitative PCR showing significant suppression in IL-1 beta gene expression in ileal loops exposed to *Clostridium difficile* toxin A and GSNO (p<0.05, * and #, significantly different to PBS/Veh and TxA/GSNO, respectively). **Figure 7C** shows dose-dependent killing of human intestinal Caco-2 epithelial cells by *C. difficile* toxin A.

Figures 8A-8B show effect of GSNO on adhesion of diarrheagenic *E. coli* binding and its effect on the bacterial growth. **Figure 8A** shows that adhesion of pathogenic *E. coli* to intestinal epithelial Caco-2 cells was significantly inhibited by preincubation of bacteria with GSNO. The bacterial growth curves in **Figure 8B** show that the GSNO effects are not due to bacterial killing. Rather inhibition is due to cysteine modification of bacterial proteins by NO and/or glutathione groups.

Figure 9 shows a disease activity index demonstrates a significant protective effect of oral co-administration of GSNO (10 mg/kg/day) in the drinking water during 7 days of 5% DSS-treatment in Balb/c mice (p<0.05 on days 3-7). No disease activity is evident following oral administration of GSNO without DSS.

Figure 10 shows GSNO concentrations in human colonic tissues (Top). Biopsies from control patients with no history of IBD (non-IBD; n=29); Crohn's disease patients (CD; n=12) and ulcerative colitis patients (UC; n=2) were compared. A 7.6-fold decrease is apparent when comparing control and CD groups (p=0.013). Both groups failed the normality test and the Mann-Whitney Rank Sum Test was used for statistical analysis. Median values (25% & 75% intervals) were 182.5 nM/mg protein (95.4 & 378.8) for controls and 23.9 nM/mg protein (0.9 & 125.2) for CD which included both involved and non-involved

tissues. (Below) QrtPCR analysis for human γ-GT expression. Patient groups include control patients without IBD (CONT; n=27); normal (CD-N; n=15) and inflamed (CD-I; n=31) colonic biopsies from Crohn's disease; normal (UC-N; n=11) and inflamed (UC-I; n=25) colonic biopsies from ulcerative colitis. Dunn's Test was used for all pair-wise comparisons following rank-based ANOVA. Median values and statistical differences
5 compared with non-inflamed Crohn's disease biopsies (CD-N) are highlighted.

Figure 11 shows S-nitrosothiol immunofluorescence in UC. Colonic epithelial SNO membrane immunoreactivity (green) and counterstained DAPI positive nuclei (blue) [mag x400]. The white arrows indicate the position of the apical epithelial brush border membrane.

Figure 12 is a 2-D gel showing S-nitrosylated proteins from a patient biopsy (Left). Spots
10 can be excised for identification and analysis of biotin-cysteine modifications by mass spectrometry. S-nitrosylated claudin-2 is indicated by the arrow. A hypothetical structural organization of the claudin family showing the WWCC motif (right).

Figure 13 is a biotin-switch blot for GSNO-treated cells showing several novel S-nitrosylated protein species (left). Streptavidin-pull down of biotinylated proteins following GSNO treatment
15 of Caco-2 cells demonstrates that the tight junction protein claudin-2 can be identified following immunoblotting with an anti-claudin 2 specific antibody (right).

Figure 14 shows transepithelial resistances in transfected Caco-2 cells. GSNO-induced 197% and 138% increases in pCMV6-CLD-2 and pCMV6-empty transfected cells, respectively.

Figure 15 is a regression analysis showing colonic claudin-2 and IL-13 mRNA expression
20 in control and IBD patient biopsies.

Figure 16 shows IL-13 treatment (10 ngml) of Caco-2 cells. (Left) Relative claudin-2 mRNA expression after 6 hrs, and (right) transepithelial resistances (TER) after 24 hrs of IL-13 stimulation +/- 50 μM GSNO.

Figure 17 shows GSNO (10 mg/kg/day) drug-mediated alleviation of pain symptoms in a
25 rat acetic-acid induced irritable bowel disease.

DETAILED DESCRIPTION OF THE INVENTION

The present invention demonstrated that (i) mucosal barrier integrity required enteric glial cell
30 functions *in vivo*, (ii) soluble factors generated by enteric glia induced barrier properties in epithelia *in vitro*, (iii) nitrosoglutathione present in enteric glial cell-conditioned media was a potent inducer of barrier properties in epithelia *in vitro, and (iv)* nitrosoglutathione maintained mucosal barrier function and protected the intestine against inflammation following genetic disruption of enteric glia *in vivo* or in non-inflamed human intestine from patients with Crohn's disease, or in infectious disease models of diarrheal disease or in a model of
35 functional bowel dysfunction/irritable bowel disease.

Under normal conditions, epithelial surfaces provide a highly selective permeability barrier that prevents the passage of toxic proinflammatory molecules from the external milieu into the submucosa and systemic circulation. Loss of this barrier integrity allows transmucosal access to normally excluded luminal substances *e.g.* endotoxin and microbes, and this may lead to inflammation and tissue injury. Loss of
40 epithelial barrier function has been implicated in a wide range of inflammatory disorders, including

inflammatory bowel disease, diabetic retinopathy and pulmonary edema. The pathogenesis of epithelial barrier dysfunction is poorly understood, although chronic tissue inflammation and the release of reactive oxygen species are implicated in the loss of tight-junction integrity.

The findings presented herein show that nitrosoglutathione was a potent barrier-inducing factor produced by enteric glia. CNS-astroglia were producers and secretors of GSH and GSNO. Although a role for nitrosoglutathione in epithelial barrier protection has not been demonstrated, studies have shown it to maintain vascular integrity either by acting as a low-dose NO donor to endothelial cells and/or by altering the function of key molecular regulators of barrier function via cGMP-independent transnitrosylation *e.g.* of the p50 subunit of NFκB or cyclooxygenase-2. Moreover, NO-signaling may alter epithelial barrier function. The conflicting literature on this subject may be roughly divided into protective (Zech et al., 2998; Gookin et al., 2004) or disruptive (Lee et al., 2005; Gorodeski et al., 2000; Han et al., 2004) effects on mucosal integrity. These contradictory findings may relate to, and be explained by, the dose-dependent effects of nitrosoglutathione herein. At low μM concentrations GSNO promoted epithelial barrier function, while at higher μM-to-mM concentrations that could potentially be achieved under pathologic conditions where the inducible-form of NOS (iNOS) is highly expressed in glia (Chatterjee et al., 2000; Dringen et al., 2000), nitrosoglutathione disrupted epithelial barrier-integrity.

Furthermore, nitrosoglutathione significantly promoted human intestinal mucosal barrier function in Crohn's disease patients, but not in intestinal tissues from individuals without inflammatory bowel disease. This tissue-specificity may relate to the observation that the enteric glial cell network is particularly disrupted in non-inflamed Crohn's disease intestinal mucosa, and that as a consequence, tissue nitrosoglutathione concentration levels may be lower in these patients.

The findings discussed herein indicate that enteric glial cell disruption may constitute a primary cause of epithelial permeability disorders leading to tissue inflammation, and that exogenous nitrosoglutathione treatment might prevent mucosal barrier failure in this context. The identification of nitrosoglutathione as a peripheral glial cell-derived, small, soluble molecule that can protect epithelial-barrier integrity via parenteral delivery represents a therapeutic mediator in the treatment of human inflammatory barrier disorders, especially inflammatory bowel disease, but also of other functional bowel disorders such as irritable bowel disease, gastroparesis, diabetes associated gut dysfunction and infectious diarrheal disease.

Clostridium difficile, a Gram-positive non-invasive toxigenic bacterium, is a frequent cause of antibiotic-associated diarrhea and colitis in humans and animals. *C. difficile* infection affects millions of patients each year and is a cause of infectious diarrhea and colitis in hospitalized patients. Toxigenic strains of *C. difficile* release two large protein exotoxins, toxin A (307 kDa) and toxin B (279 kDa). Both toxins possess cytotoxic activities including the dissagregation of actin microfilaments and cell rounding. The cellular mechanism of action of toxins A and B involves their binding to carbohydrate cell surface receptors, and following endocytosis, disruption of the actin cytoskeletal network mediated by modification of the Rho family of GTPases. This involves the glucosylation of specific threonine residues which is catalyzed by glucosyltransferase domains, utilizing UDP-glucose as substrate, located at the N-termini of both toxins A and B.

Since administration of toxin A into the intestine of animals causes diarrhea, tissue necrosis and an intense inflammatory infiltrate, the present invention examined the effect of S-nitrosoglutathione in a

murine model of Clostridium difficile toxin A-induced enterocolitis. The present invention demonstrates that GSNO reduces (i) intestinal fluid secretion due to loss of mucosal barrier function, (ii) intestinal inflammation and pathology, (iii) the ability of toxin A to reduce epithelial barrier function and cell rounding by inactivating the toxin function. Moreover, GSNO-mediated inactivation of enterotoxin is facilitiated by inositol phosphate or phytic acid co-factors.

Escherichia coli O157:H7 is a human pathogen that colonizes the intestine causing a diarrheal syndrome characterized by a copious bloody discharge which can be fatal due to acute kidney failure (hemolytic-uremic syndrome). Curli-expressing thin aggregative fimbriae, which are rarely reported in *E. coli* O157:H7 compared with other pathogenic *E. coli* strains, reportedly bind eukaryotic extracellular matrix proteins as well as to enhance the formation of *E. coli* O157:H7 biofilms on inert surfaces. Biofilm formation may increase *E. coli* O157:H7 survival and would likely result in protection against many environmental conditions.

The present invention also examined the effect of GSNO on the adhesion of *E. coli* O157:H7 to intestinal epithelial cells and demonstrates that GSNO promoted epithelial barrier function and prevented intestinal inflammation by reducing bacterial binding to intestinal epithelial cells. This finding was also evident with EPEC, ETEC, EAEC and Shigella flexneri infections.

The present invention is directed to a method of treating intestinal inflammation and dysfunction in an individual, comprising: administering a pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual, thereby treating the intestinal inflammation in the individual. Additionally, the administration of the compound may also restore the intestinal mucosal barrier function. Additionally, the administration of the compound may also inhibit the visceral pain and irritable bowel in the individual. An individual who may benefit from this method is not limited to but may include one with an inflammatory bowel disease, irritable bowel disease, gastroparesis, intestinal food allergies, infective colitis, toxin and drug induced barrier disease, ischemic reperfusion and bowel disease, CNS trauma and blood-brain barrier dysfunction, pulmonary edema, microbial infection, diabetic retinopathy or diabetes.

Furthermore, the inflammatory bowel disease is not limited to but may include necrotizing enterocolitis, Crohn's disease, ulcerative colitis, ischemic bowel disease and infective colitis. The infective colitis may be caused by Clostridium difficile, E. coli O157:H7, EPEC, ETEC, EAEC and Shigella. Specifically, the examples of the thiol reactive compounds that may be used in such a method may include but are not limited to S-nitrosoglutathione, *S*-nitrosoglutathione diethyl ester, S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosocysteine (CGSNO), reduced glutathione, and hydrogen sulfide, Furthermore, the compound may be administered orally, subcutaneously, intravenously, topically or by inhalation.

The present invention is also directed to a method of regulating permeability of mucosal epithelia, comprising: contacting an epithelial cell of the mucosal epithelia with a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups such that the contact induces expression of one or more than one epithelial tight-junction associated protein, transnitrosylation of one or more than one epithelial tight-junction associated proteins, transnitrosylation of toxin released by toxigenic bacteria, inhibition of binding of pathogenic bacteria to mucosal epithelial cells or a combination thereof, thereby regulating the permeability of the mucosal epithelia. The epithelial tight-junction associated protein

that may be targeted by the compound includes but may not be limited to zonula occludens-1 (ZO-1), occludin, or claudin-2. Furthermore, examples of the toxigenic bacteria may include but are not limited to Clostridium difficile and examples of pathogenic bacteria may include but are not limited to diarrheagenic E.coli species and shigella. Examples of the compounds that may be used in this method are the same as described supra.

5 The present invention is further directed to a method of treating inflammatory bowel disease in an individual, comprising: administering a pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual, where the administration restores the intestinal mucosa barrier function, attenuates inflammation of the gut, or a combination thereof, thereby treating the disease in the individual. Additionally, the compound may restore 10 the intestinal mucosal barrier function by inducing expression of one or more than one epithelial tight-junction associated proteins, transnitrosylation of one or more than one epithelial tight-junction associated proteins or a combination thereof. Examples of the epithelial tight-junction associated proteins, the compounds that may be administered and the route of administration are the same as described supra. Additionally, the compound may restore the intestinal mucosal barrier function by inhibiting interleukin-13-induced barrier dysfunction via 15 down regulating insulin-receptor associated signaling pathways.

The present invention is still further directed to a method of treating Clostridium difficile toxin-induced colitis in an individual, comprising: administering pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual, where the administration inactivates the toxin, restores intestinal mucosal barrier function, 20 attenuates tissue inflammation or a combination thereof, thereby treating Clostridium difficile toxin-induced colitis in the individual. Examples of the compounds that may be administered and the route of administration are described supra. Additionally, inhibition of enterotoxin activity is further facilitated by addition of inositol phosphate/phytic acid.

The present invention is also directed to a method of treating EHEC, EPEC, ETEC, EAEC 25 and Shigella-induced colitis in an individual, comprising: administering pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual, where the administration prevents bacterial binding to the epithelium, restores the intestinal mucosal barrier function, attenuates tissue inflammation or a combination thereof, thereby treating the infective-induced disease in the individual. Examples of the compounds that may be administered and the route 30 of administration are described supra.

As used herein, the term, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" or "other" may mean at least a second or more of the same or different claim element or components thereof. As used herein, the term "contacting" refers to any suitable method of bringing the 35 epithelial cell into contact with the compound described herein. *In vitro* or *ex vivo* may be achieved by exposing the above-mentioned cell to the compound in a suitable medium. In vivo may be achieved by any of the routes that are routinely used in the art. As used herein, the term "microbial infection" refers to any infection that is caused by virus, fungus or parasite. As used herein, the term "intestinal food allergies" includes but is not limited to coeliac disease. As used herein, the term "mucosal epithelia" refers to mucosal

epithelia of the intestine, the lung, the kidney, genital tract and skin, but also encompasses the blood-brain barrier because of functional similarities.

As used herein, the term "compound" means a molecular entity of natural, semi-synthetic or synthetic origin that regulates, maintains or restores the mucosal barrier function, attenuates inflammation, reduces gut pain, or a combination thereof. The compound described herein can be administered independently, either systemically or locally, by any method standard in the art. The routes of administration are not limited to but include oral, subcutaneous, intravenous, topical or nasal route. Dosage formulations of the compound described herein may comprise conventional non-toxic, physiologically or pharmaceutically acceptable carriers or vehicles suitable for the method of administration and are known to an individual having ordinary skill in this art.

The compound described herein may be administered independently or in combination with another drug or compound that is routinely used to treat other symptoms of that specific disorder and may comprise one or more administrations to achieve, maintain or improve upon a therapeutic effect. It is well within the skill of an artisan to determine dosage or whether a suitable dosage of the composition comprises a single administered dose or multiple administered doses. An appropriate dosage depends on the subject's health, the restoration of tissue barrier function or attenuation of inflammation or inhibition of pain, the route of administration and the formulation used.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. One skilled in the art will appreciate

EXAMPLE 1

Intestinal permeability in vivo

Conventional 12 week-old GFAP-HSVtk transgenic mice were utilized after genotyping a tail biopsy by PCR analysis as described (Bush et al., 1998). Mice were housed in a controlled temperature and humidity environment (12 hr light/dark cycles) and were allowed access to food and water *ad libitum*. For conditional ablation of EGC, ganciclovir (GCV; Roche) was administered *s.c.* at a rate of 100 mg/kg/day using mini-osmotic pumps (Alzet) for 7 days. Intestinal tissues were snap-frozen in liquid nitrogen and stored at -80°C. For the *in vivo* permeability studies, mice were fasted overnight and gavaged with 60 mg/100g body weight of FITC-dextran (4.4 kD at 80 mg/ml) or 20 mg/100g body weight fluorescein-5,-6-sulfonic acid (478 daltons; Molecular Probes) as described (Furuta et al., 2001). Cardiac puncture was performed after 4 hrs for serum analysis. For non-invasive intestinal transit studies, mice were given a 0.1 ml gavage of PeakFlow™ Infrared flow cytometry reference beads (770 nm emission, 6 μm diameter, 2% solid; Molecular Probes) and scanned at various times using a Li-Cor Odyssey infrared scanner (Li-Cor Biosciences). GSNO was administered *i.p.* at 10 mg/kg/day.

EXAMPLE 2

Patients and Ussing chamber studies

Three patients with Crohn's Disease and healthy controls (aged 18-75 years) were prospectively included. Two Crohn's disease patients were receiving azathioprine and one received steroids.

All patients underwent endoscopy. Colonic biopsy specimens were obtained from non-inflamed histologically normal mucosa of Crohn's disease patients. Mucosal biopsies were taken from patients free of organic intestinal disease who underwent flexible sigmoidoscopy or colonoscopy as part of a gastroenterological work up.

5 Colonic biopsies were mounted in 1.5 ml mini-Ussing chambers (TBC-Transcellab, Paris, France) with a 2mm diameter (mucosal surface exposed: $3.14mm^2$). After mounting, each half chamber was filled with culture medium (DMEM containing 0.1% FCS) and was continuously oxygenated with O_2/CO_2 (5/95%). After 15 min of equilibration, 150 μl of media was removed from the apical chamber and was replaced with an equal volume of media containing 1 mg/ml FITC-Inulin (4kDa; Sigma-Aldrich). At the same 10 time, GSNO or vehicle was added to the basolateral chamber to a concentration of 100 μM. The fluorescent intensity in the basolateral chamber was then measured immediately after this procedure to determine baseline fluorescence. Fluorescent intensity in the basolateral chamber, reflecting paracellular transit from the lumenal surface, was measured for 2h at regular time-intervals in a Victor and was normalized to the initial basal level value.

15

EXAMPLE 3

Myeloperoxidase Assay

Neutrophil myeloperoxidase (MPO) activity is an indicator of tissue inflammation. Bowel segments (100-250 mg) were homogenized in 1 ml HTAB buffer and centrifuged at 20,000g for 10 min at 4^0C. 20 Pellets were resuspended in 1 ml HTAB buffer containing 1% hexadecyltrimethlammonium to negate pseudoperoxidase activity. MPO activity was measured in supernatants following 3 cycles of sonication, freezing and thawing. After centrifugation at 40,000g for 15 min at 4^0C, supernatants (10 μl) were mixed with 90 μl of potassium phosphate buffer containing 0.167 mg/ml O-dianiside dihydrochloride and 0.0005% hydrogen peroxide. Activity was measured every 2 minutes for 20 minutes at 450 nm.

25

EXAMPLE 4

Real-time PCR

Total RNA was extracted from frozen tissues, treated with 1 U DNAse I and reversed transcribed (Gene Amp RNA-PCR Kit). Real-time multiplex rt-PCR was performed using TaqMan probes 30 conjugated with FAM, VIC, Texas Red or Cy5. Alternatively rtPCR reactions were run with SYBR Green PCR Master-Mix for 40 cycles on a Chromo4 detector (BioRad Ltd) (94°C for 2 min; 94°C for 1 min; 60°C for 1min; 72°C for 1min; repeat step 2-to-4 for 40 cycles; 72°C for 10 min). Primer sets for TNFα (Forward 5`-ATGAGCACAGAAAGCATGATC-3` (SEQ ID NO. 1), Reverse 5`-TACAGGCTTGTCACTCGAATT-3`) (SEQ ID NO. 2); IL-1β (Forward 5`-TTGACGGACCCCAAAAGATG-3` (SEQ ID NO. 3), Reverse 5`-35 AGAAGGTGCTCATGTCCTCA-3`) (SEQ ID NO. 4); IL6 (Forward 5`-TGGAGTCACAGAAGGAGTGGCTAAG-3` (SEQ ID NO. 5); Reverse 5`-TCTGACCACAGTGAGGAATGTCCAC-3`) (SEQ ID NO. 6); GFAP (Forward 5'-GAGGAGGAGATCCAGTTCTTAAGGA-3' (SEQ ID NO. 7), Reverse 5'-GCCTCGTATTGAGTGCGAATC-3' (SEQ ID NO. 8); probe 5'-CCAGACCTCACAGCGGCCCTGA-3') 40 (SEQ ID NO. 9); nNOS Forward 5`-GGGAAACTCTCGGAGGAGGA-3` (SEQ ID NO. 10), Reverse 5`-

TGAGGGTGACCCCAAAGATG-3' (SEQ ID NO. 11), probe 5'-CGTGGTACCGGTTGTCATCCCCTCAG-3')(SEQ ID NO. 12). Samples were normalized against commercial GAPDH or 18S rRNA primers and probes (Applied Biosystems) and relative expression levels were calculated as described previously (Livak and Schmittgen, 2001).

5

EXAMPLE 5

Cell culture

All reagents were obtained from Sigma or Gibco unless otherwise stated. Astroglial cell cultures included the astrocytoma cell line C6 grown in M199 media supplemented with 10% fetal calf serum, 10 50 U/ml penicillin and 50μg/ml streptomycin in a 95% air: 5% CO_2 mixture. EGC cell cultures included a transformed rat-myenteric plexus derived line and primary non-transformed murine or rat myenteric EGC prepared as described previously (Bannerman *et al* 1988). NIH 3T3 murine fibroblasts, human intestinal adenocarcionoma Caco-2, and canine kidney-derived MDCK epithelial cells were all grown in DMEM. Stable MDCK cell lines expressing constitutively active or dominant-negative forms Rac-1, Rho-A or cdc-42 under 15 the control of the tetracycline-repressible transactivator were grown in media supplemented with or without 20ng/ml doxycycline (DC) as described (Jou et al., 1998). Cell viability was assessed using a standard MTT cytotoxicity assay.

Conditioned EGC media was prepared by incubating cells overnight in media containing no or 1% FCS followed by centrifugation at 14000 rpm for 5 min. Ultrafiltration of conditioned media was 20 prepared using a 1kDa cut-off filter in a stirred ultrafiltration cell (Millipore). For co-culture experiments, 5 x 10^4 Caco2, HT29 or MDCK cells were seeded on $Cellagen_{TM}$ membrane dics (collagen I, 14 mm diameter, ICN Biomedicals). EGC, C6 astrocytes or 3T3 fibroblasts were then seeded at an equal density either on the underside of the filter or in the bottom of the wells to avoid any possibility of cell contact. Culture media was changed every 24 hours after taking electrical tissue resistance measurements using a Volt-ohm meter (EVOM, 25 World Precision Instruments). To inhibit endogenous NOS activity, 1kDa CM was prepared from EGC incubated with 100 μM L-NAME. Alternatively, EGC-derived 1kDa CM was incubated with 20U/ml carboxypeptidase Y, 1mM glutathione-dependent formaldehyde dehydrogenase (NAD^+/$NADP^+$), or 1mM dithiothreitol for 2 hr at 37^0C.

30

EXAMPLE 6

FPLC and mass spectrometry

Samples were injected into a BioCad Sprint (Applied Biosystems) system. A Superdex Peptide HR10/30 column (Pharmacia Biotech) was used to separate peptides in the molecular weight range 100-to-7000 Da. A flow rate of 1 ml/ml was used to resolve 1kDa CM in 20 mM phosphate buffer (pH7.4) 35 containing 25 mM NaCl and active fractions were tested in culture with Caco2 cells. Mass spectrometry was performed by the Proteomics Core at UTMB using an ESI interface. Both the electronspray needle and the skimmer were operated at ground potential, whereas the electrospray chamber and metalized entrance of the glass capillary were operated at –3.5 kV in the positive ion mode. Acetonitrile (5%) and 0.01% acetic acid were added to HR10/30 active fractions prior to infusion into the electrospray ion source. Compounds were 40 analysed for *m/z* signals.

EXAMPLE 7

Western blot analysis

Epithelial cell cultures were washed with PBS and Triton X-soluble and -insoluble protein fractions were prepared (Chen et al., 2002). Confluent epithelial cell monolayers grown on filters were washed three times with ice-cold PBS, lysed in Triton X-100 buffer (1% Triton X-100, 100 mM NaCl, 10 mM HEPES, pH 7.6, 2 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 4 mM sodium orthovanadate, 40 mM sodium fluoride), and then passed through a 21-gauge needle ten times. The lysates were then centrifuged at 15,000 x g for 30 min at 4 °C. The resulting supernatant was considered the Triton X-100-soluble fraction. The pellet was then solubilized in Triton X-100 buffer containing 1% SDS using an ultrasonic disintegrator, cleared by centrifugation at 15,000 x g for 5 min at 4 °C, and referred to as the Triton X-100-insoluble fraction. The protein concentration of each sample was quantified by the Bradford method. Samples were electrophoresed through a 4-20% gradient SDS polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore). After 1 h blocking (Tris-buffered saline, 0.1% Tween 20 (TBS-T), 1% BSA), the blots were incubated overnight at 4°C with the first antibody layer diluted in blocking buffer. After washing in TBS-T, the membrane was incubated with an appropriate second antibody diluted in blocking buffer for 1 h at room temperature. Bands were then detected by ECL kit (Amersham) or by infrared imaging on an Odyssey imager (Li-Cor Biosciences). Immunoblots were stripped with 62.5 mM Tris (pH 6.8), 2% SDS containing 10 mM 2-ME at 50°C for 1 h. Western blots were analysed densitometrically with NIH Image 1.6 software or using Li-Cor software. Blots were subsequently hybridized with antibodies against tight junction-associated proteins to allow direct comparison in the same samples. All Western blots are representative for at least three experiments carried out.

EXAMPLE 8

Immunostaining

At the indicated time, cells were washed three times with Dulbeco's phosphate-buffered saline (PBS; Life Technologies) and were fixed in acetone for 1 min at 20°C. Thereafter, the cells were blocked with 1:20 PBS diluted normal donkey serum (blocking solution) for 30 min at 20°C and incubated with blocking solution-diluted first antibodies for 1 h at room temperature using anti-E-cadherin, -GFAP, -nNOS, -ZO-1, -claudin-1 and -occludin antibodies (Zymed Ltd) or 10 μg/ml TRITC-phalloidin (Molecular probes). Results discussed herein are as mean values ± S.E.M. Statistical significance was determined using Krushal-Wallis one way analysis of variance on ranks for analysis of multiple groups, Rank Sum Test for comparison of two groups with a non-gaussian population or Student's t-test after demonstrating normality using the Kolmogorov-Smirnow test for normality on SigmaStat 2.0 software. $P < 0.05$ was considered statistically significant.

EXAMPLE 9

Enteric glial cell-disruption promoted intestinal permeability in vivo

To determine whether enteric glial cell disruption altered intestinal barrier properties *in vivo*, mucosal paracellular permeability to small fluorescent probes (FITC-dextran (4.4 kDa) and fluorescein-5,-6-

sulfonic acid (478 Da)) was measured in GFAP-HSVtk transgenic mice and in control non-transgenic littermates receiving ganciclovir (GCV) treatment or vehicle control for 7 days. This time-point was identified with substantial disruption of enteric glia but little intestinal pathology in transgenic mice.

Real-time fluorescent imaging of mice receiving an intra-gastric gavage of soluble PeakFlow™ infrared reference beads demonstrated that the probe reached the distal small intestine within 4 hours (Fig. 1A). Significantly elevated serum FITC-dextran and fluorescein-sulfonic acid measurements were recorded in ganciclovir-treated GFAP-HSVtk transgenic mice (Fig. 1B). Mucosal permeability was therefore elevated at an early stage of enteric glial cell disruption, the latter being demonstrated by a reduced GFAP mRNA accumulation in ileal tissues (Fig. 1C). Furthermore, enhanced mucosal permeability due to local production of proinflammatory cytokines was ruled out by demonstrating no significant increases in TNFα, IL-1β and IL-6 mRNA accumulation or an elevation in neutrophil myeloperoxidase activity at this time point after GCV (Fig. 1D). In addition, no histological signs of intestinal pathology, tissue inflammation, neuronal damage, abnormal neuropeptide expression or intestinal transit were observed at this time point. Thus, mucosal barrier integrity depends on functions provided directly by enteric glia.

EXAMPLE 10

In vitro modeling of enteric glia-induced mucosal barrier function

Whether epithelial cell properties associated with barrier functions were directly modified *in vitro* by exposure to enteric glia or to glial-derived soluble factors, in a manner analogous to effects on cerebral endothelia by astrocyte cultures were also examined. To do so, primary or transformed enteric glia that retained characteristic cellular markers in culture, such as GFAP, S-100β, glutamine synthetase and nerve growth factor receptor p75 (Figs. 2A-2E) were used. Intestinal Caco-2, HT29 and kidney-derived MDCK epithelial cells were co-cultured with these enteric glia.

Confluent epithelia grown on Cellagen$_{TM}$ disc membranes in co-culture with peripheral enteric glia demonstrated significantly greater transepithelial resistances of up to two-fold greater than cells grown in media alone or in co-culture with 3T3 fibroblasts (Fig. 3A). Macromolecular permeability to FITC dextran (4.4 kDa) and fluorescein sulfonic acid (478 Da) was significantly diminished in epithelia co-cultured with glia (Fig. 3B), and this correlated with a significant up regulation of tight-junction-associated proteins zonula occludens-1 (ZO-1) and occludin (Fig. 3C), as well as increased F-actin accumulation to lateral membranes (Fig. 3D).

Conditioned media prepared from enteric glia or C6 astrocytes also significantly elevated transepithelial resistance in Caco-2 monolayers (Fig. 4A). Ultra-filtration of conditioned media demonstrated significant barrier-inducing activity in the smaller than 1kDa cut-off fraction, but not in the greater than 1kDa fraction (Fig. 4A). This smaller than 1 kDa ultrafiltrate significantly increased transepithelial resistance by up to 3-fold when applied to the basolateral membrane compartment but not to the apical domain (Fig 4B), indicating that the epithelial basolateral cell surface is the primary membrane site involved in the enteric glial-derived activity. This induction of transepithelial resistance occurred rapidly within 12 hours. On the other hand, quantitative rtPCR demonstrated that intestinal brush border dipeptidyl peptidase IV mRNA accumulation was not significantly induced after 24 hours, thereby indicating that the glial barrier-inducing

activity is not manifested by promoting cellular differentiation (1.13-fold increase; p=0.47, Rank Sum Test; n=3). This enteric glial-derived barrier-inducing activity is referred to as barrier-inducing factor.

EXAMPLE 11

5 Molecular characterization of glial-derived barrier inducing factor (BIF) as GSNO

Although enteric glia express barrier-inducing factors, notably TGF-β and GDNF, it was possible to eliminate these because they exceeded the size limitation of the 1kDa cut-off for the *BIF*-enriched fraction. Further purification of *BIF* activity using Superdex Peptide HR10/30 size exclusion chromatography demonstrated a transepithelial resistance-inducing activity in a 300-to-500 Da fraction (Fig. 4C) that was 10 analyzed using electron spray mass spectrometry. Several peaks were identified, which included oxidized and nitrosylated forms of the anti-oxidant peptide glutathione (GSH) (Fig. 4D). Synthetic forms of these compounds were screened in culture and *s*-nitrosoglutathione demonstrated an induction in transepithelial resistance (Fig. 5A) that was not recapitulated using oxidized or reduced forms of GSH (Fig. 5B). S-nitrosoglutathione did not induce transepithelial resistance in a dose-dependent manner but promoted resistance 15 at lower μM concentrations and this effect was reversed at higher concentrations. (Fig. 5A)

A requirement for the cellular formation of *s*-nitrosoglutathione is transnitrosylation of GSH from reactive nitric oxide (NO) intermediates catalyzed by nitric oxide synthetase isoforms (NOS), or from the cellular expression of the enzyme ceruloplasmin that serves as a NO^+ donor to the thiolate on GSH. Rat primary enteric glial cell cultures constitutively expressed eNOS and low levels of nNOS as observed by 20 quantitative-multiplex rtPCR, western blot and immunohistochemistry. Following serum-starvation, enteric glia also expressed the inducible-form of NOS (iNOS). Blocking NOS activity in enteric glia using N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) inhibited *BIF* activity in Caco-2 epithelial cells. Lastly, transepithelial resistances were reduced when 1kDa conditioned media was pretreated with carboxypeptidase, glutathione-dependent formaldehyde dehydrogenase or thiol-reducing dithiothreitol. Thus, GSNO was mediating 25 a significant component of enteric glial-derived *BIF* activity in culture.

EXAMPLE 12

S-nitrosoglutathione promoted mucosal barrier function in vivo

To test the physiological relevance of *s*-nitrosoglutathione in protecting mucosal barrier 30 function *in vivo*, GFAP-HSVtk transgenic mice given ganciclovir were treated with a daily intra-peritoneal dose of *s*-nitrosoglutathione (10 mg/kg) or vehicle for 7 days, and intestinal permeability was then measured using orally gavaged fluorescein-sulfonic acid. GSNO treatment inhibited the increased intestinal permeability caused by enteric glial cell ablation in transgenic mice, demonstrating that *s*-nitrosoglutathione had a protective effect on mucosal barrier function *in vivo*. Parentally administered *s*-nitrosoglutathione also protected 35 transgenic mice from intestinal inflammation. By day 11 of ganciclovir, vehicle-treated transgenic mice displayed fulminant jejuno-ileitis, whereas the intestine in GSNO-treated animals showed only minor inflammatory lesions. These pathologic differences were reflected in the percentage mortality, which showed a significant improvement in GSNO-treated animals. No adverse effects were observed in non-transgenic mice receiving vehicle or *s*-nitrosoglutathione for 14 days. Thus, the barrier-inducing effects of *s*-nitrosoglutathione 40 delay the onset of inflammation in this transgenic model of enteric glial cell-ablation.

Enteric glia are abundant in the intestinal mucosa where they are closely juxtaposed to the epithelium. As such, they are ideally placed to secrete molecules that interact with the epithelial basolateral membrane and alter mucosal permeability. To examine whether *s*-nitrosoglutathione directly altered tight-junction expression in intestinal epithelial cells, ZO-1 immunofluorescence on Caco-2 cells exposed to 10 μM *s*-nitrosoglutathione for up to 48 hours was performed. ZO-1 immunolabelling was confined to the apical tight-junction region and provided a clear cellular outline (Fig. 6A). Although no obvious visual differences were observed in the pattern of ZO-1 immunolabelling in control and GSNO-treated cells, mRNA accumulation and protein expression in the triton-x insoluble cytoskeletal fraction were elevated following GSNO-exposure. (Fig. 6B). Thus, at physiological concentrations *s*-nitrosoglutathione promoted epithelial-barrier function by inducing ZO-1 gene expression and by promoting its association with the cytoskeleton. GSNO promoted ZO-1 expression *in vivo*. Following ablation of enteric glia in transgenic mice, apical epithelial ZO-1 immunolabelling was disrupted. Parenteral administration of GSNO (10 mg/kg) to transgenic mice attenuated the disruption of ZO-1 expression.

To examine whether *s*-nitrosoglutathione was also able to promote human intestinal mucosal barrier function, human colonic biopsies in Ussing chambers were exposed to synthetic compound and the paracellular flux of FITC-Inulin (4 kDa) was measured sequentially over 2 hours. GSNO at 100 μM did not alter base-line mucosal permeability in histologically normal colon from control patients without inflammatory bowel disease, demonstrating that this concentration was not acutely toxic to epithelial cells *in situ*. Non-involved (histologically normal) Crohn's disease colonic biopsies demonstrated a trend towards higher mucosal permeability as compared to controls (Fig. 6C). This increased mucosal paracellular flux in Crohn's disease patients was significantly inhibited following addition of *s*-nitrosoglutathione to the basolateral compartment. Thus, *s*-nitrosoglutathione was able to restore mucosal barrier function in non-inflamed colon from patients with Crohn's disease, an inflammatory bowel disease with an associated permeability disorder.

EXAMPLE 13

S-nitrosoglutathione promotes intestinal barrier disruption and prevents intestinal inflammation in a model of Clostridium difficile toxin A-induced enterocolitis

Administration of toxin A into the intestine of animals causes diarrhea accompanied by tissue necrosis and an intense inflammatory infiltrate. Therefore, the effect of GSNO on Clostridium difficile toxinA-induced enterocolitis was examined in a murine model. Briefly, the murine model comprised of CD1 male mice (Charles River Laboratories, Wilmington, MA) weighing 30–35 g that had free access to food and water in a 12-h light/dark cycle. Mice were acclimated to these conditions at least 7 days before the experiment. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and ileal loops (3–4 cm) were prepared and injected with buffer alone or with the GSNO (100 uM), in a volume of 200 μl. After 20 min, toxin A (10 μg in PBS) or PBS alone was injected intraluminally, and animals were sacrificed 4 h later by CO_2. Ileal loop fluid was collected and centrifuged at 50,000 x *g* for 15 min. Ileal loops were excised and weighed, and length was measured. Fluid secretion was expressed as the loop weight-to-length ratio (mg/cm). Ileal tissue samples were quick frozen for immunohistochemical analysis and for protein determination. GSNO significantly reduced (i) intestinal fluid secretion due to loss of mucosal barrier

function, (ii) intestinal inflammation and pathology, (iii) the ability of toxin A to reduce epithelial barrier function and cell rounding by inactivating the toxin function, most likely via transnitrosylation (Figures 7A-7C).

5

EXAMPLE 14

S-nitrosoglutathione inhibits adhesion of Escherichia coli to intestinal cells

GSNO inhibits adhesion of diarrheagenic E. coli to intestinal epithelial cells (Fig. 8A). Bacteria were incubated with GSNO prior to contact with Caco-2 intestinal epithelial cells. The growth curves in Fig. 8B demonstrates that effects against the bacteria are not due to bacterial killing, but rather cystein modification of

10 bacterial proteins.

EXAMPLE 15

S-nitrosoglutathione inhibits binding of bacteria to mucosal epithelial cells.

Strains were routinely grown in Luria-Bertani (LB) broth or on L agar at 37°C. When

15 indicated, the strains were grown in Dulbecco's modified Eagle's medium (Cellgro; Mediatech, Inc., Herndon, VA). Antibiotics (Sigma-Aldrich, Co., St. Louis, MO) were added to media at the following concentrations: kanamycin (Km), 50 μg/ml; ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 30 μg/ml; streptomycin (Sm), 100 μg/ml; tetracycline (Tc), 12.5 μg/ml; nalidixic acid (Nal), 30 μg/ml; and neomycin, 20 μg/ml in liquid and 60 μg/ml in solid media.

20 Bacterial adhesion to epithelial cells:

Caco-2 cells were seeded with 1 x 10^5 cells/well and incubated for 48 h at 37°C with 5% CO_2 in 24-well plates (Corning, Inc., Corning, NY). The cell monolayers were washed twice with phosphate-buffered saline (pH 7.4), and the infection was carried out with wild-type bacteria. Briefly, bacterial strains were grown in LB broth overnight at 37°C, the monolayers were infected with 1 x 10^7 bacteria for 3 h, and adherence

25 was evaluated qualitatively by Giemsa staining and quantitatively by plating adherent bacteria on L agar plates with an appropriate antibiotic. The results were performed in triplicate and repeated at least twice. *P* values were calculated using a paired *t* test. GSNO dose-dependently reduced the binding of EHEC, EPEC EAEC but not Salmonella to intestinal epithelial cells without affecting bacterial viability. The ability of GSNO to inhibit bacterial binding to mucosal epithelial cells promoted epithelial barrier function and prevented intestinal

30 inflammation.

EXAMPLE 16

GSNO promotes intestinal barrier function via S-nitrosylation of epithelial cells

GSNO is an endogenous *S*-nitrosylating agent that regulates several cell-signaling cascades

35 via post-translational modification of cellular proteins. This process involves the transfer of an NO^+ group to a cysteine thiol-residue forming an *S*-nitrosothiol. Therefore, cellular *S*-nitrosylation signals are distinct from classical NO-sensitive cGMP-dependent regulation.

S-nitrosylation reactions regulate specific physiologic and pathophysiologic signaling cascades by directly modifying transcription and/or protein function. *S*-nitrosylation of different protein species

40 can alter their function. For example, cyclooxygenase, thioredoxin, CFTR and $p21^{ras}$ activities are increased,

whereas NFkB, caspase and methionine adenosyl transferase activities are inhibited. *S*-nitrosylation of protein thiols may also occur as a result of *S*-transnitrosylation by endogenous small molecular weight *S*-nitrosothiols (SNO's), notably GSNO. Generally, specific protein thiols are targeted by *S*-nitrosylation. Moreover, many bioactivities that are regulated by *S*-nitrosylation *e.g.* blood pressure and vascular tone, are stereo-selective
5 where the *S*-nitrosothiol L-isomer, but not the D-isomer is active. Both isomers release NO radicals at the same rate, which indicates the presence of stereo-specific SNO cell receptors. This stereo-selective approach may be used to characterize *S*-nitrosylation reactions. SNO can also initiate cell signaling via release of NO.

CNS-astroglia are prolific producers and secretors of GSNO. GSNO biosynthesis results from the intracellular nitrosylation of glutathione, forming an *S*-nitrosothiol with the generic structure R-S-
10 N=O. GSH is an abundant intracellular peptide and is a vital anti-oxidant. Intracellular redox reactions that generate nitrosylating species *e.g.* O_2 or transition metals are important catalysts in the formation of GSNO. Intracellular *S*-nitrosylation of GSH by CysNO and HcysNO are also important in the generation of GSNO. CysNO and HcysNO are transported into cells via the L-AT$^-$ and perhaps other carrier systems. GSNO cannot enter cells directly as it requires conversion to CysNO by γ-glutamyl transpeptidase (γ-GT) before uptake is
15 possible. Once formed, GSNO is metabolised by GSNO reductase. GSNO is also generated by reactive NO formed during nitric oxide synthetase (NOS) activity, and the interactions of NO and O_2 are promoted by their enrichment in hydrophobic membrane compartments.

Figure 9 demonstrates that GSNO confers protection against experimental dextran sodium sulphate (DSS)-induced colitis.

20

GSNO effector system deficiency in Crohn's disease and GSNO replenishment therapy

Analysis of tissue GSNO concentrations demonstrated significantly lower levels in colonic biopsies from Crohn's disease patients when compared with controls (Fig. 10). To examine whether exogenous GSNO is also able to promote human intestinal mucosal barrier function, human colonic biopsies
25 in Ussing chambers were exposed to GSNO and measured the paracellular flux of FITC-Inulin (4 kDa) sequentially over 2 hours.

In contrast to biopsies from control patients, intestinal permeability in Crohn's disease patients was significantly inhibited following addition of GSNO (100 μM) to the basolateral compartment. In addition, the response was rapid (within 1 hour) indicating that GSNO induces barrier function via *S*-
30 transnitrosylation signals, possibly by post-translational modification of tight junction proteins themselves.

S-nitrosylation of intestinal epithelial cells and tight junction proteins

Immunohistochemistry performed with an anti-*S*-nitrosocysteine (SNO-Cys) polyclonal antibody demonstrated that in inflamed ulcerative colitis colon, epithelial cell membranes contain highly *S*-
35 nitrosylated protein species (Fig. 11). To demonstrate that tight junction proteins are directly regulated by *S*-nitrosylation, the biotin-switch assay was applied to screen for *S*-nitrosylated proteins in (*i*) patient biopsies and (*ii*) in Caco-2 cells following incubation with 100 μM GSNO for 30 min. The biotin-switch assay involves several steps to identify *S*-nitrosylated cysteine residues. First, sulfhydryl groups are blocked; SNO groups are then reduced to free sulfhydryl groups, and finally, these new sulfhydryl groups are labeled with
40 biotin to allow subsequent detection by western blot analysis and mass spectrometry following streptavidin-

precipitation. An example of a streptavidin-pull down assay of biotinylated proteins from inflamed colon is demonstrated in Figure 12. Mass spectrometry analysis of the *S*-nitrosylated proteome has identified several post-translationally modified proteins, tentatively including the tight junction protein claudin-2 (Fig. 12). Claudins are integral membrane proteins that have four hydrophobic transmembrane domains. The two extracellular loops are involved in homophilic and/or heterophilic protein interactions that impart barrier function and ion selectivity to the tight junction. The WWCC motif, W(17-22)-W-X(2)-C-X(8-10)-C, within the first extracellular loop is highly conserved among claudin family members.

Although the second extracellular loop does not contain any cysteine residues, highly conserved aromatic and hydrophilic residues within this loop appear to be important in regulating claudin-claudin interactions and tight junction strand formation (Fig. 12). *S*-nitrosylation is also governed by a consensus motif. An acid-base consensus sequence is observed in proteins where the modified cysteine residue has been defined. The most important characteristic of this motif is an Asp (D) or Glu (E) following the target cysteine. In order to identify potential cysteine targets of *S*-nitrosylation, clustal W alignment of the first extracellular loop region of human claudin family proteins that are known to be expressed in the intestine was performed.

CLD1 PQWRIYSYAGDNIVTA--QAMYEGLWMSCV-SQSTGQIQCKVFDS--LLNLSST-LQ (SEQ ID NO 13)

CLD2 PSWKTSSYVGASIVTA--VGFSKGLWMECA-THSTGITQCDIYST--LLGLPAD-IQ (SEQ ID NO 14)

CLD3 PMWRVSAFIGSNIITS--QNIWEGLWMNCV-VQSTGQMQCKVYDS--LLALPQD-LQ (SEQ ID NO 15)

CLD4 PMWRVTAFIGSNIVTS--QTIWEGLWMNCV-VQSTGQMQCKVYDS--LLALPQD-LQ (SEQ ID NO 16)

CLD7 PQWQMSSYAGDNIITA--QAMYKGLWMDCV-TQSTGMMSCKMYDS--VLALSAA-LQ (SEQ ID NO 17)

CLD8 PQWRVSAFIENNIVVF--ENFWEGLWMNCV-RQANIRMQCKIYDS--LLALSPD-LQ (SEQ ID NO 18)

CLD12 PNWRKLRLITFNRNEK-NLTVVYTGLWVKCA—RYDGSSDCLMYDTTWYSSVDQLDLR (SEQ ID NO 19)

CLD15 SYWRVSTVHG-NVITT--NTIFENLWFSCA-TDSLGVYNCWEFPS--MLALSGY-IQ (SEQ ID NO 20)

CLD18 DMWSTQDLYDNPVT---SVFQYEGLWRSCV-RQSSGFTECRPYFT--ILGLPAM-LQ (SEQ ID NO 21)

CLD20 PNWKVNVDVDSNIITA--IVQLHGLWMDCT-WYSTGMFSCALKHS--ILSLPIH-VQ (SEQ ID NO 22)

This WWCC motif-region of the first extracellular loop contains two conserved cysteine residues that are known to be functionally important in mediating tight junction characteristics. As is evident from the primary claudin amino acid sequence alignment below, only claudin-2 (CLD2) possesses the predicted *S*-nitrosylation motif on the second conserved cysteine-residue (underlined). Claudin-2 *S*-nitrosylation by

GSNO was confirmed by biotin-switch analysis of Caco-2 cells (Fig. 13). Streptavidin-precipitation demonstrated that several proteins are *S*-nitrosylated by GSNO *in vitro* and subsequent immunoblotting identified claudin-2 as post-translationally modified.

The stability of *S*-nitrosylated target cysteine residues varies to a great extent. Cytoplasmic and mitochondrial-exposed cysteine residues are rapidly denitrosylated by effectors enzymes that regulate cellular signal transduction pathways through stimulus-coupled *S*-nitrosylation. However, membrane-associated and extracellular cysteine modifications have a much longer half-life (minutes to hours) by virtue of a favorable microenvironment that promotes *S*-nitrosothiol stability. Thus, it is feasible that *S*-nitrosylation of functionally important cysteine residues that are either membrane-associated or are located in the extracellular domain of tight junction proteins is a 'reasonably stable' post-translational modification. Thus, the effects that GSNO has on intestinal barrier function where claudin-2 expression levels are elevated either following (*i*) transient transfection or (*ii*) IL-13 stimulation was examined.

Transient transfection

The feasibility of using transient transfection to study claudin-2 pore-forming activity is examined. Initially, Caco-2 cells are transfected in solution using Amexa-based technology and cells are seeded at high density on collagen filters. 50% of cells strongly express the transgene after 3 days in culture under such conditions. Transepithelial resistances in the transfected Caco-2 cells is measured. GSNO-induced 197% and 138% increases in pCMV6-CLD-2 and pCMV6-empty transfected cells, respectively (Fig. 14).

IL-13 stimulation

IL-13 activates several signaling cascades, including the STAT6 and PI3-kinase pathways. IL-13 induced barrier dysfunction in colonocytes is mediated by the PI3-kinase, implicating recruitment of the insulin receptor substrate family. Whether GSNO also inhibited IL-13 induced barrier dysfunction was examined in Caco-2 cells. Claudin-2 mRNA expression is significantly up regulated in colonic biopsies from IBD patients, and correlated positively with IL-13 mRNA expression (Fig. 15).

Moreover, IL-13 (10 ng/ml) induced a 5-fold elevation in Caco-2 cell claudin-2 mRNA expression after 6 hrs in culture and triggered a 25% decrease in TER after 24 hrs (Fig. 16). GSNO inhibited the induction of claudin-2 expression by % and restored TER to % of controls.

S-nitrosylation signals that protect the intestine from interleukin-13 (IL-13) induced barrier dysfunction

IL-13 induced intestinal barrier dysfunction is mediated by the PI3-kinase implicating recruitment of the insulin receptor substrate family. Elevated expression of pore-forming claudin-2 represents an effector arm for this barrier dysfunction. The present invention demonstrated that GSNO protects the intestinal barrier from IL-13 stimulation *in vitro*. As the insulin receptor substrate family is known to be rapidly *S*-nitrosylated and degraded by GSNO, *S*-nitrosylation of insulin receptor substrate-1 (IRS-1), protein kinase B/Akt and claudin-2 represents a likely signaling mechanism for this inhibition.

Biotin-switch assay to identify S-nitrosylated protein targets

The biotin-switch assay is performed away from direct sunlight essentially with the following modifications. Cell lysates are diluted to 1 mg/ml with HEN buffer (250 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine, pH 7.7); 100 μl of 25% w/v SDS and 20 μl of 10% (v/v in DMSO) *S*-methylmethane thiosulfonate (MMTS) is added per ml (blocking of free thiol at 50°C for 20 min). Proteins are then precipitated to remove excess MMTS by addition of one vol of acetone for 20 min at -20°C. After centrifugation at 4000xg for 5 min, the pellet is washed 3x with 70% acetone and re-suspended in 850 μl HEN buffer containing 1% SDS, 50 μl of sodium ascorbate in HEN buffer (giving 5 or 50 mM final concs, optimized to detect endogenous and over-expressed proteins) and 100 μl of biotin-HPDP (2.5 mg/ml in DMSO) are added to label (biotinylate) *S*-nitrosylated proteins. Labeling is performed in the dark for 60-90 min; proteins are then precipitated with 50% acetone (to remove excess biotin-HPDP), the pellets washed with 70% acetone and re-suspended in 25 mM Hepes, 1 mM EDTA, 1%SDS (HEN/10+SDS). Proteins are then precipitated and re-suspended in 250 μl HEN/10+SDS and 750 μl neutralization buffer (25 mM Hepes, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 7.7). This solution is added to 30-50 μl streptavidin agarose beads washed with neutralization buffer and tumbled at 4°C o/n. After precipitation, the beads are centrifuged at 200xg for 10 s and 50 μl supernatant is reserved for immunoblotting. Beads are then washed 5x (25 mM Hepes, 600 mM NaCl, 1 mM EDTA, 0.5% TritonX-100). Protein is eluted with 45 μl elution buffer (25 mM Hepes, 100 mM NaCl, 1 mM EDTA, 100 mM 2-mercaptoethanol) for 30 min at room temp. 6x loading buffer is added to the effluent and the samples are separated on 10% SDS-PAGE and immunoblotted for IRS-1, protein kinase B/Akt, and claudin-2.

Detection of S-nitrosylated targets in intestinal tissues

Intestinal tissues or biopsies are rinsed with PBS pH 7.4, containing 100 μM DTPA until free of blood. Tissues are then homogenized in 1 ml lysis buffer using a polytron. After centrifugation at 20,000 xg for 15 min, the supernatant is diluted to 1 mg/ml in HEN buffer and the biotin-switch assay is performed as described above.

The present invention demonstrated that the L-isomer of GSNO is active in Crohn's disease patients undergoing treatment with azathioprine and steroids but not in control patients without IBD. While the biological half-life of NO is short (<1 sec), its functionality can be prolonged, and in many regards more discretely modulated, when it reacts with low-molecular weight and protein-bound thiols to form *S*-nitrosothiols from which NO subsequently can be re-released. In the case of GSNO, *in vivo* release of NO occurs primarily via *S*-transnitrosylation of other protein thiol species. The half-life of GSNO following *in vivo* systemic administration in rats is approximately 20 min in the presence of activated T and B lymphocytes that express high levels of surface g-GT At 10 mg/kg/day, GSNO concentrations should remain high enough to promote intestinal barrier function for at least 2 hrs. This assumes similar degradation rates in the intestinal mucosa and a 5 μM lower sensitivity limit as demonstrated for transformed intestinal epithelial cell lines *in vitro*. It is possible that the barrier-inducing sensitivity is lower in non-transformed intestinal epithelial cells *in vivo*, as has been shown for GSNO-mediated intestinal microvascular protection at >30 nM/kg. Furthermore, two independent reports on experimental EAE and IRBP-mediated autoimmune disease have demonstrated significant protective effects following oral GSNO administration at 0.3 mg/kg/day. In this

regard, enzymatic cleavage of GSNO by g-GT is a key regulatory mechanism that promotes bio-activation (not degradation) of this signaling pathway.

EXAMPLE 17

5 Effects of GNSO in rat irritable bowel disease model

Rats with acetic-acid (AA) induced irritable bowel disease were administered 10 mg/kg/day GSNO in a vehicle. Controls were AA rats with vehicle only and healthy rats with saline-vehicle and saline-GNSO. Figure 17 demonstrates that GNSO alleviated pain symptoms in the AA rats.

10 The following references may have been cited herein:

Abbott NJ et al. *Nat. Reviews* 7, 41-53 (2006).
Ballabh P et al. *Neurobiol. Dis.* 16, 1-13 (2004).
Bannerman et al., *Brain Res.* 440, 99-108 (1988).
Buhner S, *et al. Gut* 55,342-347 (2006).
15 Bush TG, *et al. Cell* 93, 189-201 (1998).
Cabarrocas J, et al. T *Glia* 41, 81-93 (2003).
Chatterjee et al., *Glia* 29, 98-101 (2000).
Chen ML et al. *J. Biol. Chem.* 277, 4247-4254 (2002).
Cornet A, *et al. PNAS* 98, 13306-13311 (2001).
20 Do KQ, *et al. Neurochem.* Int. 29, 213-224 (1996).
Dringen R, et al. *Eur. J. Biochem.* 267, 4912-4916 (2000).
Farhadi et al., *J. Gastroenterol. Hepatol.* 18, 479-489 (2003).
Felenski et al., *Curr. Eye Res.* 30, 949-957 (2005).
Fries W, *et al. Am. J. Gastroenterol.* 100, 2730-2736 (2005).
25 Furuta GT, *et al. J. Exp. Med.* 193, 1027-1034 (2001).
Gaston BM, et al. *Mol. Intervention* 3, 253-263 (2003).
Gershon MD and Rothman TP *Glia* 4, 195-204 (1991).
Gershon M.D. and Bursztajn S. J. *Comp. Neurol.* 180, 467-488 (1978).
Gookin JL, *et al. Am. J. Physiol.* 287, G571-G581 (2004).
30 Gorodeski GI. *Am. J. Physiol.* 278, C942-C952 (2000).
Guihot G, *et al. Amino Acids* 18, 229-237 (2000).
Han et al., *Shock* 21, 261-270 (2004).
Hogg N. *Ann. Rev. Pharmacol. Toxicol.* 42, 585-600 (2002).
Hollander D. *J. Physiol. Pharmacol.* 54, 183-190 (2003).
35 Hurst RD and Fritz IB. *J Cell Physiol* 167:81-88 (1996).
Jaffrey et al., *Nat. Cell. Biol.* 3, 193-197 (2001).
Janzer RC and Raff MC. *Nature* 325, 253-257 (1987).
Jiang et al., *Exp. Brain Res.* 162, 56-62 (2005).
Jou TS and Nelson WJ. *J. Cell Biol.* 142, 85-100 (1998).
40 Khan M, *et al. J. Cerebral Blood Flow Metab.* 25, 177-192 (2005).

Kim SF et al. *Science* 310, 1966-1970 (2005).
Laroux FS, *et al. Acta Physiol. Scand.* 173, 113-118 (2003).
Lee SW, *et al. Nat. Med.* 9, 900-906 (2003).
Lee et al., *Biol. Reprod.* 73, 458-471 (2005).
5 Livak KJ and Schmittgen TG. *Methods* 25, 402-408 (2001).
Minich T, *et al. J. Neurochem.* 97, 373-384 (2006).
Nazli A, *et al. Am. J. Path.* 164, 947-957 (2004).
Neunlist M, *et al. Am. J. Physiol. Gastrointest Liver Physiol.* 2006 Jan 19.
Neunlist M, *et al. Am. J. Physiol.* 285, G1028-G1036 (2003).
10 Parmantier E, *et al. Neuron* 23, 713-724 (1999).
Pavlick KP, *et al. Free Rad. Biol. Med.* 33, 311-322 (2002).
Que LG, *et al. Science* 308, 1618-1621 (2005).
Savidge TC, *et al. Microecol. Ther.* 28, 81-92 (1999).
Schonhoff CM, *et al. PNAS* 103, 2404-2409 (2006).
15 Suenaert P, *et al. Inflam. Bowel Dis.* 11, 667-673 (2005).
Yang et al., *Free Rad. Biol. Med.* 36, 1317-1328 (2004).
Zech JC, *et al. Invest. Ophthalmol. Vis. Sci.* 39, 1600-1608 (1998).

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are 20 incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A method of treating intestinal inflammation in an individual, comprising:

administering a pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual, thereby treating the intestinal inflammation in the individual.

2. The method of claim 1, wherein said administration restores the intestinal mucosal barrier function.

3. The method of claim 1, wherein the individual has an inflammatory bowel disease, irritable bowel disease, intestinal food allergies, infective colitis, toxin and drug induced barrier disease, ischemic reperfusion and bowel disease, CNS trauma and blood-brain barrier dysfunction, pulmonary edema, microbial infection, diabetic retinopathy or diabetes.

4. The method of claim 3, wherein the inflammatory bowel disease is necrotizing enterocolitis, Crohn's disease, ulcerative colitis, ischemic bowel disease or infective colitis.

5. The method of claim 4, wherein the infective colitis is caused by Clostridium difficile or diarrhegenic E.coli.

6. The method of claim 1, wherein the compound is *s*-nitrosoglutathione, *s*-nitrosoglutathione diethyl ester, S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosocysteine (CGSNO), reduced glutathione, or hydrogen sulfide.

7. A method of regulating permeability of mucosal epithelia and blood-brain barrier, comprising:

contacting an epithelial cell of the mucosal epthelia or blood-brain barrier associated endothelia with a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups such that said contact induces the expression of one or more than one epithelial tight-junction associated protein, transnitrosylation of one or more than one epithelial tight-junction associated protein, transnitrosylation of toxin released by toxigenic bacteria, inhibition of binding of pathogenic bacteria to mucosal epithelial cells or a combination thereof, thereby regulating the permeability of the mucosal epithelia.

8. The method of claim 7, wherein the epithelial tight-junction associated protein is zonula occludens-1 (ZO-1), claudin-2 or occludin.

9. The method of claim 7, wherein the toxigenic bacteria is Clostridium difficile.

10. The method of claim 7, wherein the pathogenic bacteria is EHEC, EPEC ETEC, EAEC or Shigella.

11. The method of claim 7, wherein the compound is *s*- nitrosoglutathione, *s*-
5 nitrosoglutathione diethyl ester, S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosocysteine (CGSNO), reduced glutathione, or hydrogen sulfide.

12. A method of treating inflammatory bowel disease in an individual, comprising:

administering a pharmacologically effective amount of compound comprising a nitric oxide
10 group and/or a chemical entity that modifies cysteine thiol groups to the individual, wherein said administration restores the intestinal mucosal barrier function, attenuates inflammation of the colon, or a combination thereof, thereby treating the inflammatory bowel disease in the individual.

13. The method of claim 12, wherein the compound restores the intestinal mucosal barrier
15 function by inducing expression of one or more than one epithelial tight-junction associated protein, transnitrosylation of one or more than one epithelial tight-junction associated protein, or a combination thereof.

14. The method of claim 13, wherein the epithelial tight-junction associated protein is
20 zonula occludens-1 (ZO-1), claudin-2 or occludin.

15. The method of claim 12, wherein the compound is nitrosoglutathione, *s*-nitrosoglutathione diethyl ester, S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosocysteine (CGSNO), reduced glutathione, or hydrogen sulfide.

25

16. A method of treating irritable bowel disease or functional bowel disorders in an individual, comprising:

administering a pharmacologically effective amount of compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual, wherein said
30 administration alleviates gut pain, restores the intestinal mucosal barrier function, attenuates inflammation of the colon, or a combination thereof, thereby treating the functional boweld disease in the individual.

17. The method of claim 16, wherein the compound reduces functional bowel disease by attenuating visceral pain, inhibiting intestinal inflammation, restoring intestinal mucosal barrier function by
35 inducing expression of one or more than one epithelial tight-junction associated protein, transnitrosylation of one or more than one epithelial tight-junction associated protein, or a combination thereof.

18. The method of claim 16, wherein the compound is nitrosoglutathione, *s*-nitrosoglutathione diethyl ester,. S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosocysteine (CGSNO),
40 reduced glutathione, or hydrogen sulfide.

19. A method of treating Clostridium difficile toxin-induced colitis in an individual, comprising:

administering pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual, wherein said administration inactivates the toxin, restores intestinal mucosal barrier function, attenuates tissue inflammation or a combination thereof, thereby treating Clostridium difficile toxin-induced colitis in the individual.

20. The method of claim 19, wherein the compound is nitrosoglutathione, s-nitrosoglutathione diethyl ester, S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosocysteine (CGSNO), reduced glutathione, or hydrogen sulfide.

21. The method of claim 19, further comprising:

administering an inositol phosphate or phytic acid formulation.

22. A method of treating EHEC, EPEC, ETEC, EAEC, or Shigella -induced diarrheal disease in an individual, comprising:

administering pharmacologically effective amount of a compound comprising a nitric oxide group and/or a chemical entity that modifies cysteine thiol groups to the individual, wherein said administration prevents bacterial binding to the epithelium, restores the intestinal mucosal barrier function, attenuates tissue inflammation or a combination thereof, thereby treating the infectious dierrheal disease in the individual.

23. The method of claim 22, wherein the compound is nitrosoglutathione, s-nitrosoglutathione diethyl ester, S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosocysteine (CGSNO), reduced glutathione, or hydrogen sulfide.