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Gastrointestinal, Hepatobiliary, and Pancreatic Pathology

Targeting α-7 Nicotinic Acetylcholine Receptor in the Enteric Nervous System

A Cholinergic Agonist Prevents Gut Barrier Failure after Severe Burn Injury

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We have previously shown that vagal nerve stimulation prevents intestinal barrier loss in a model of severe burn injury in which injury was associated with decreased expression and altered localization of intestinal tight junction proteins. α-7 Nicotinic acetylcholine receptor (α-7 nAchR) has been shown to be necessary for the vagus nerve to modulate the systemic inflammatory response, but the role of α-7 nAchR in mediating gut protection remained unknown. We hypothesized that α-7 nAchR would be present in the gastrointestinal tract and that treatment with a pharmacological agonist of α-7 nAchR would protect against burn-induced gut barrier injury. The effects of a pharmacological cholinergic agonist on gut barrier integrity were studied using an intraperitoneal injection of nicotine 30 minutes after injury. Intestinal barrier integrity was examined by measuring permeability to 4-kDa fluorescein isothiocyanate-dextran and by examining changes in expression and localization of the intestinal tight junction proteins occludin and ZO-1. Nicotine injection after injury prevented burn-induced intestinal permeability and limited histological gut injury. Treatment with nicotine prevented decreased expression and altered localization of occludin and ZO-1, as seen in animals undergoing burn alone. Defining the interactions among the vagus nerve, the enteric nervous system, and the intestinal epithelium may lead to development of targeted therapeutics aimed at reducing gut barrier failure and intestinal inflammation after severe injury. *(Am J Pathol 2012, 181:478–486; http://dx.doi.org/10.1016/j.ajpath.2012.04.005)*

Gut barrier failure, a sequela of several medical conditions, results in significant illness and disability.[1] The gut barrier and the immune system work in concert to form a defense against the potentially toxic elements found in the gut lumen.[2] Several studies have demonstrated that different disease processes, including inflammatory bowel disease, severe burns, traumatic brain injury, and necrotizing enterocolitis, lead to a certain degree of intestinal barrier injury, which is characterized by marked changes within the gut epithelium.[3–5] After injury or inflammatory insult, the injured gut releases proinflammatory mediators into the mesenteric lymph, activating inflammatory and endothelial cells and causing distant organ injury.[6,7] Secondary infections, which are common among injured patients, can further damage gut barrier integrity and drive the inflammatory response by stimulating already primed inflammatory cells, also causing distant organ injury.[8,9] Developing therapies that prevent gut barrier failure may have wide-reaching applications in treatment of patients after injury or those with inflammatory disorders of the intestine.

Harnessing the intrinsic anti-inflammatory mechanisms of the nervous system may be an ideal therapeutic strategy to limit injury and inflammation. The mechanism by which the parasympathetic nervous system regulates systemic inflammation via the vagus nerve continues to be defined.[10,11] Vagus nerve stimulation (VNS) has been shown to decrease circulating proinflammatory cytokine levels in an animal model of sepsis.[12] VNS exerts its systemic anti-inflammatory effects through its ability to decrease production of splenic proinflammatory media-

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tors after injury via α-7 nicotinic acetylcholine receptor (α-7 nAchR).[13] This mechanism has been confirmed in studies showing that the protective, anti-inflammatory effects of VNS are lost in mice that either lack α-7 nAchR or have undergone splenectomy.[13,14]

Enteric glial cells (EGCs), a cell type within the enteric nervous system, are key regulators of intestinal barrier function.[15] EGCs are phenotypically similar to astrocytes of the central nervous system, a population of cells long recognized as key regulators of the blood-brain barrier and also involved in responding to inflammation.[16] EGCs secrete numerous molecules when activated, several of which improve gut barrier function and limit mucosal inflammation.[17] Because of their barrier-protective properties, EGCs have recently been the focus of research designed to understand the pathway by which the enteric nervous system maintains homeostasis and controls inflammation.[18–20]

We have recently demonstrated the ability of VNS to limit gut barrier injury in a model of severe burn injury.[18,21,22] Our data suggest that VNS protects the intestine by modulating the response to injury at the level of the gut tissue, rather than by altering systemic inflammation, and that VNS is associated with increased activation of EGCs.[18] Although gut protection in this model appears to be independent of splenic cytokine production, the potential role of α-7 nAchR in mediating the gut-protective effects of VNS has not previously been defined. In this series of experiments, we postulated that α-7 nAchR would be present in the gastrointestinal tract, and that treatment with a pharmacological agonist of α-7 nAchR would protect against burn-induced gut barrier injury.

Materials and Methods

Caco-2 Cell Culture

Caco-2 intestinal epithelial cells were obtained from ATCC (Manassas, VA) and were grown in a 5% CO_2 humidified atmosphere at 37°C. Cells were grown in confluent monolayers on 12-well Transwell plates (80,000 cells per well) in Dulbecco's modified Eagle's medium with high glucose (Gibco; Invitrogen-Life Technologies, Carlsbad, CA). The medium was changed daily. Enterocyte differentiation medium containing penicillin G, 10% fetal bovine serum, streptomycin, and 1% nonessential amino acids was added on day 3. Cells were removed on day 5 for further analysis with Western blot or confocal microscopy.

Enteric Glial Cell Culture

Enteric glial cells (ATCC) were grown in 5% CO_2 humidified atmosphere at 37°C in 12-well plates with 30,000 cells per well. Cells were grown in Dulbecco's modified Eagle's medium, which was changed daily for 3 days. On day 3, the medium was replaced with serum-free medium. Cells were removed on day 4 for further analysis.

In Vitro *Permeability*

Caco-2 cells and EGCs were cultured alone, or in coculture before the addition of Cytomix (IFN-γ, TNF-α, and IL-1β, 500 ng/mL; Sigma-Aldrich, St. Louis, MO). Selected cells were also incubated in the presence of nicotine (0.1 mmol/L). After incubation with Cytomix for 24 hours, 200 μL of 4-kDa fluorescein isothiocyanate (FITC)-dextran (10 mg/mL; Sigma-Aldrich) was added to the apical well of the Transwell insert. Next, 100-μL aliquots of Dulbecco's modified Eagle's medium were removed from the basal chamber after 4 hours. Fluorescence was measured in a fluorescence spectrophotometer (SpectraMax M5; Molecular Devices, Sunnyvale, CA) and compared with a standard curve using known concentrations of FITC-dextran.

Burn Model

Male BALB/c mice weighing 20 to 24 g were obtained from the Jackson Laboratory (Sacramento, CA; Bar Harbor, ME). Animals were exposed to a 12-hour light-dark cycle and had access to food and water ad libitum. Mice were first anesthetized using inhaled isoflurane and then their dorsal fur was removed with an electric clipper. Mice were placed into a template designed to estimate 30% total body surface area and were exposed to a severe, 7-second steam burn. After injury, animals were injected with 1.5 mL of normal saline with buprenorphine subcutaneously in a nonburned region for pain control and fluid resuscitation. Animals were then returned to their cages to recover from anesthesia. Control animals received a sham burn treatment; they were placed under general anesthesia, underwent clipping of their dorsal fur, and received an injection of normal saline with buprenorphine, but were not burned. A separate cohort of animals underwent an intraperitoneal injection of nicotine (400 μg/kg; Sigma-Aldrich) in PBS 30 minutes after burn injury. A group of animals also underwent splenectomy before injury, performed by midline laparotomy incision and ligation of the splenic artery and vein at the splenic hilum using silk suture. Animal experiments were approved by the University of California Animal Subjects Committee.

Histological Evaluation

Sections of distal ileum were harvested 4 hours after injury, fixed in formalin, and embedded in paraffin. Sections of paraffin were stained with H&E by staff at the University of California, San Diego Histology Core facility. Sections were then viewed under light microscopy. Images were reviewed by a pathologist (Dr. Paul Wolf) blinded to the experimental groups and were scored for evidence of intestinal injury based on three randomly selected fields per specimen ($n = 4$ animals per group). Scoring was on a scale from 0 to 4, as follows: 0, normal, no injury; 1, mild, focal edema; 2, moderate, diffuse swelling and necrosis of the villi; 3, severe, diffuse pathology of the villi; 4, major, widespread injury with massive inflammatory cell infiltration.

Intestinal Permeability Assay

Intestinal permeability to 4-kDa FITC-dextran was measured at 4 hours after injury ($n = 5$ animals per group). Animals were placed under general anesthesia with in-

haled isoflurane. A midline laparotomy incision was made and the small intestine was exposed. A 5-cm segment of distal small intestine was isolated between silk ties. The isolated segment of gut was injected with 25 mg of FITC-dextran that had been dissolved in 200 μL of PBS. The bowel was returned to the abdominal cavity, and the skin was closed with silk suture. Animals were kept under anesthesia for 30 minutes, at which point blood was obtained via cardiac puncture. Blood was placed in heparinized tubes and centrifuged at 10,000 $\times$ *g* for 10 minutes to obtain the plasma. FITC-dextran concentration was measured using a SpectraMax M5 (Molecular Devices) fluorescence spectrophotometer; sample fluorescence was compared with a standard curve using known concentrations of FITC-dextran.

Confocal Microscopy

Distal ileum was harvested 4 hours after injury and stored in optimal cutting temperature compound at −80°C (n = 4 animals per group). Sections of gut were cut 10 μm thick and fixed on glass slides using 3.7% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 minutes. Sections were then washed with PBS and incubated overnight with tight junction protein zonula occludens protein 1 (ZO-1) or occludin (Zymed, Carlsbad, CA) antibody. Sections were then exposed to Alexa Fluor 488 (Invitrogen-Life Technologies) goat anti-rabbit antibody in 1% BSA for 1 hour. Images were obtained using an Olympus FluoView laser scanning confocal microscope with exposure-matched settings (Advanced Software version 1.6; Olympus). Paracellular space measurement between ZO-1 staining from adjacent epithelial cells was obtained by measuring the distance between fluorescence within an equivalent region of interest for each gut section, using Living Images software version 4.2 (Caliper Life Sciences, Hopkinton, MA). Data are expressed as distance in micrometers (± SEM).

Immunoblotting

Cells obtained from *in vitro* culture were lysed in 4% SDS for Western blot analysis. Cultured cells were also incubated with the nicotinic cholinergic antagonist α-bungarotoxin (20 nmol/L; Sigma-Aldrich), which served as a negative control for α-7 nAchR immunoblotting.[23] Segments of distal small intestine were harvested 4 hours after injury, snap-frozen in liquid nitrogen, and stored at −80°C. Tissue samples (n = 5 animals per group) were homogenized in tissue protein extraction reagent combined with 1% protease inhibitor and 1% phosphatase inhibitor (Pierce; Thermo Fisher Scientific, Rockford, IL). Intestinal and liver tissue were homogenized and the supernatant was collected after centrifugation at 10,000 $\times$ *g*. The protein concentration of each sample was determined using a Pierce protein concentration assay kit (Thermo Fisher Scientific). Equivalent concentrations of tissue were placed in SDS sample buffer and boiled. Proteins were separated using PAGE with Tris-glycine gradient gels (Invitrogen-Life Technologies). Gel membranes were blocked in 5% bovine serum albumin in Tris-buffered saline/Tween, then incubated overnight with the primary antibody against occludin (Zymed), claudin-1 (Invitrogen-Life Technologies), or α-7 nAchR. Membranes were then exposed to anti-rabbit IgG secondary antibody (Cell Signaling Technology, Danvers, MA) for 1 hour before application of a Pierce SuperSignal West Pico chemiluminescent kit (Thermo Fisher Scientific, Rockford, IL). Luminescence was detected using a Xenogen IVIS Lumina imaging system (Caliper Life Sciences, Hopkinton, MA). Pixel density was measured using UN-SCAN-IT gel analysis software version 5.1 (Silk Scientific, Orem, UT).

qPCR

Tissues were preserved in RNAlater (Qiagen, Valencia, CA) and stored at −80°C. RNA from 2 to 3 mg of tissue was extracted using an RNeasy mini kit (no. 74104; Qiagen) and eluted in 50 μL RNase-free water. Synthesis of cDNA was performed using an iScript cDNA synthesis kit (no. 170−8891; Bio-Rad Laboratories, Hercules, CA). Quantitative PCR was performed using Qiagen QuantiTect primers and a SYBR Green master mix (Cat. No. 204143) in a Bio-Rad iQ5 thermal cycler with assay for ZO-1 (QT00493899; Qiagen) against GAPDH (QT01658692; Qiagen). Relative fold expression was determined using the Bio-Rad iQ5 optical system software version 2.0.

Statistical Evaluation

Data are expressed as means ± SEM. Statistical significance between groups was obtained using analysis of variance with Bonferroni correction. A P value of <0.05 was considered to be statistically significant.

Results

α-7 nAchR Is Present on in Vitro *Intestinal Epithelial Cells and Enteric Glial Cells*

First, we analyzed whether α-7 nAchR is present on cultured intestinal epithelial cells. Caco-2 cells, a human intestinal epithelial cell line were grown in confluent monolayers. Cells were harvested and immunoblotted to confirm the presence of α-7 nAchR in this intestinal epithelial cell line (Figure 1A). Western blot of cultured EGCs also confirmed the presence of α-7 nAchR. Localization of α-7 nAchR was visualized by staining Caco-2 cells and EGCs and then imaging under confocal microscopy (Figure 1B). Confocal microscopy showed that the receptor distributed diffusely in cultured EGCs and Caco-2 cells. These results confirm that α-7 nAchR is located on both intestinal epithelial cells and EGCs. Both cell types are involved in mediating the gut-protective effects of VNS.

α-7 nAchR Is Expressed in the Mouse Gut in Sham and Burn Animals

Next, we determined the presence of α-7 nAchR in sham-treatment and burn-injury animals. In both groups, at 4 hours after the initial thermal insult sections of gut were

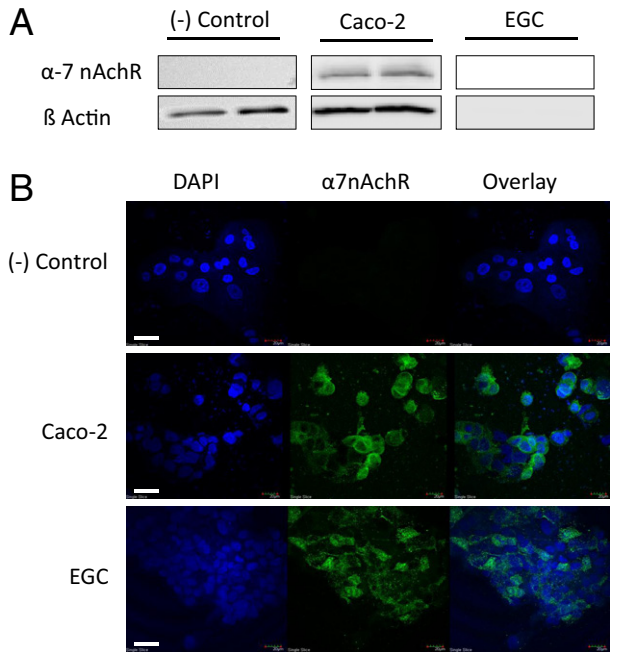

Figure 1. α-7 nAchR is expressed on cultured intestinal epithelial cells and enteric glial cells (EGCs). **A:** Caco-2 cells were grown as confluent monolayers and harvested to measure the presence of α-7 nAchR using Western blot. α-7 nAchR expression was also identified on cultured EGCs. **B:** Cultured Caco-2 cells and EGCs were stained for α-7 nAchR (green) and viewed under confocal microscopy. DAPI (blue) was used as a nuclear counterstain. Scale bar = 20 μm.

obtained to measure α-7 nAchR protein expression using Western blot. α-7 nAchR was found in the stomach, small intestine, and colon (Figure 2). There was no staining for α-7 nAchR in liver tissue, in accord with previous report.[24] There was no difference in α-7 nAchR expression between sham-treatment and burn-injury animals at 4 hours after burn treatment. α-7 nAchR is present in the gastrointestinal tract of the BALB/c mice used in this model and is therefore a candidate receptor to participate in the gut-protective effects of VNS.

Administration of a Pharmacological α-7 nAchR Agonist Prevents Gut Barrier Injury after Severe Burn

We administered nicotine, a pharmacological agonist of the nicotinic acetylcholine receptor, to determine whether stimulating α-7 nAchR would protect against burn-induced gut barrier injury. Animals given an intraperitoneal dose of nicotine after severe burn injury were compared with sham-treatment animals and animals injected with nicotine alone. Injection of nicotine prevented burn-induced histological gut injury (Figure 3, A and B) and improved gut barrier function after injury, as demonstrated by a marked decrease in intestinal permeability (Figure 3C). We analyzed a separate cohort of animals that underwent splenectomy before burn injury, to define whether the protective effects of nicotine administration were due to modulation of splenic inflammatory cells. The gut-protective effects of nicotine administration were maintained in splenectomized animals, suggesting that nicotine limits gut barrier injury through a spleen-independent mechanism.

Treatment with a Pharmacological α-7 nAchR Agonist Prevents Burn-Induced Changes in Tight Junction Protein Expression

Adequate intestinal tight junction protein function determines intestinal barrier integrity. We measured changes in expression and localization of occludin and ZO-1 to determine whether modulation of these tight junction proteins correlated with changes in gut barrier function. Expression of ZO-1 was altered in animals after the severe burn treatment, characterized by increased paracellular space between adjacent epithelial cells observed under confocal microscopy (Figure 4). Quantification of the paracellular space between ZO-1 stained cells confirmed the significant increase in space between intestinal epithelial cells (Figure 5A). Treatment with nicotine prevented the burn-induced alterations in ZO-1 expression, localization, and increased paracellular space, with findings similar to those observed in sham-treatment animals. The protective effects of nicotine administration on ZO-1 localization were maintained in splenectomized animals. Although there was altered localization of ZO-1 resulting in increased paracellular space after severe burn injury, there was no difference in ZO-1 gene expression between groups at 4 hours after injury (Figure 5B).

Changes in occludin localization were also determined using confocal microscopy imaging. Exposure-matched confocal microscopy images were acquired, to study changes in occludin localization and expression after injury as visualized by changes in fluorescent intensity

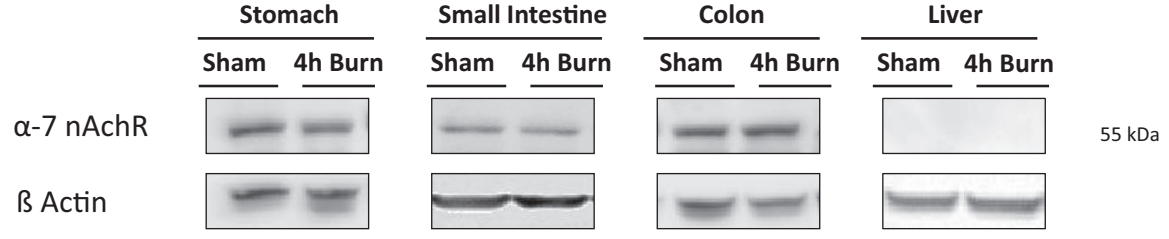

Figure 2. α-7 nAchR is present in the intestine of both sham-treatment and burn-injury animals, as indicated by band densities in Western blotting performed on protein extracted from segments of intestine and liver tissue from both groups of animals at 4 hours after sham or burn treatment. β-Actin was used as a loading control. α-7 nAchR was seen in similar concentrations in the intestine of animals in both experimental groups.

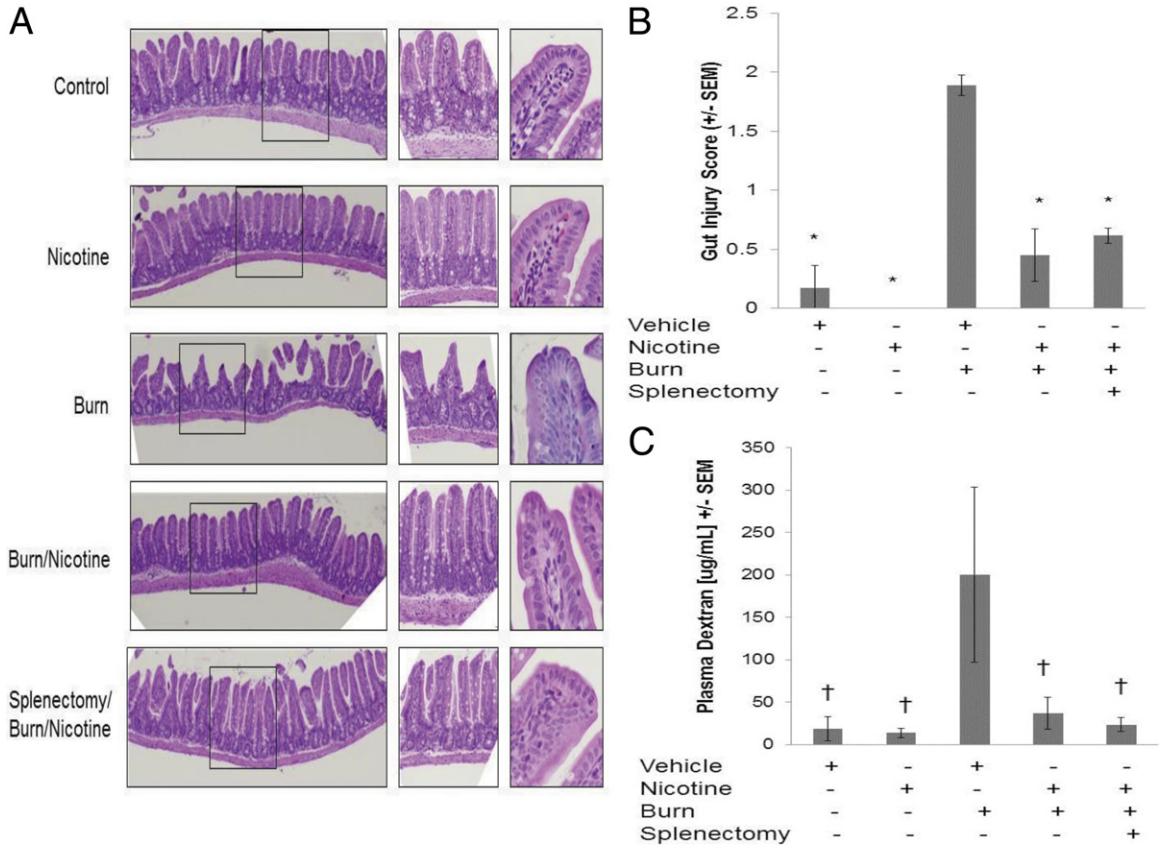

Figure 3. Nicotine protects against intestinal barrier injury. **A:** Representative micrographs of intestinal segments stained with H&E from animals 4 hours after severe burn injury. The **boxed area** is represented at two higher magnifications in the adjacent columns. Nicotine protected against burn-induced gut injury. The protective effects of nicotine were maintained in animals that underwent splenectomy, confirming that the effects are not due to modulation of systemic inflammation. Original magnification ×4, **left column**; ×20, **middle column**; ×60, **right column**. **B:** Gut injury was scored by a pathologist (Dr. Paul Wolf) blinded to the experimental groups on a scale of 0 to 4, from no injury to major, widespread injury with a massive inflammatory cell infiltration (*Materials and Methods*). **C:** Intestinal permeability to 4-kDa FITC-dextran demonstrated the intestinal barrier-protective effects of nicotine. $^{*}P < 0.05$, $^{\dagger}P < 0.01$ versus burn and vehicle.

(Figure 4). Nicotine treatment prevented the loss of occludin protein expression and the alterations in localization seen after severe burn injury. Immunoblotting confirmed the changes in protein expression seen in the confocal microscopy images, with nicotine protecting against the loss of intestinal occludin seen after severe burn injury (Figure 5C). The protective effects of nicotine were maintained in splenectomized animals, suggesting that the modulation of occludin expression was not due to a spleen-dependent mechanism. We also measured changes in intestinal claudin-1 expression after severe burn injury. There was no significant change in claudin-1 expression at 4 hours after injury (Figure 5D).

Nicotine Improves in Vivo *Intestinal Epithelial Barrier Function after an Inflammatory Insult*

To further elucidate the relative contributions of intestinal epithelial cells and EGCs in maintaining barrier function after injury, we used an *in vitro* coculture model of intestinal inflammation to assess the effects of nicotine-induced barrier protection. Stimulation with Cytomix caused increased *in vitro* permeability when added to a Caco-2-EGC coculture (Figure 6A). Treatment with nicotine prevented the Cytomix-induced increase in epithelial permeability. To determine whether the protective effects of nicotine in this *in vitro* model were due to EGCs, we repeated this experiment in Caco-2 cells in the absence of EGCs (Figure 6B). The protective effects of nicotine were lost in Cytomix-stimulated intestinal epithelial cells alone, suggesting that EGCs mediated the barrier-protective effects of nicotine.

Discussion

Previous studies have shown that VNS controls systemic inflammation by inhibiting cytokine production from inflammatory cells of the spleen.[14] Studies investigating the mechanism of the anti-inflammatory effects of VNS stimulation have focused on α-7 nAchR.[13] The anti-inflammatory effects of VNS in sepsis are lost after splenectomy, in α-7 nAchR knockout mice, and after administration of α-7 nAchR antagonists.[12,14,25] Binding of α-7 nAchR on inflammatory cells alters intracellular inflammatory signaling, resulting in decreased activation and nuclear translocation of nuclear factor-κB (NF-κB), leading to suppressed cytokine expression.[26] The vagus nerve, however, does not directly innervate the spleen, which

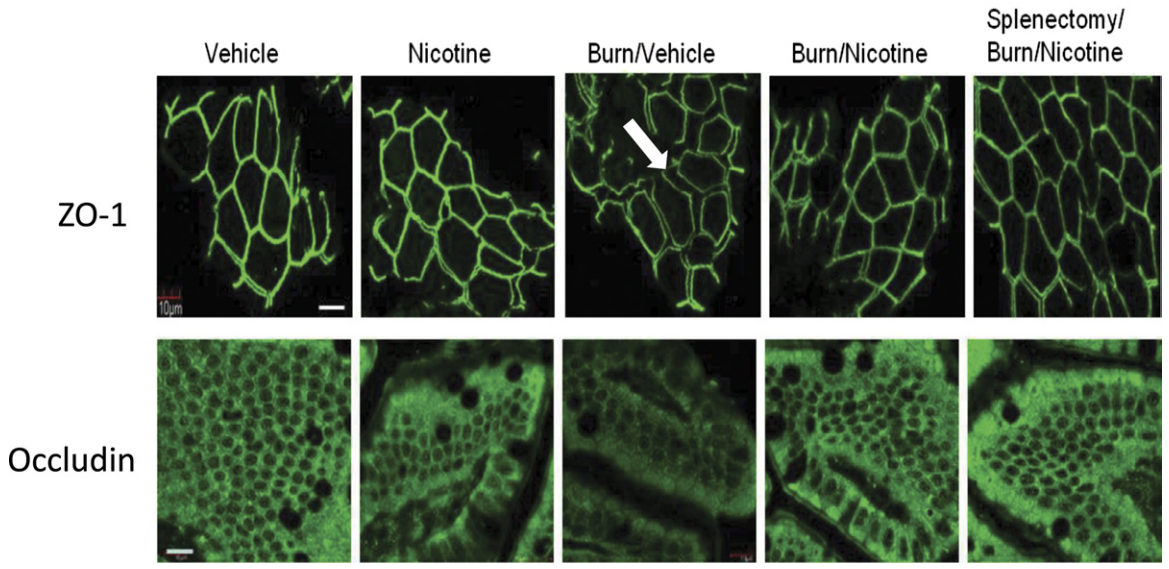

Figure 4. Nicotine prevents altered localization of intestinal tight junction protein after injury. Representative confocal microscopy images of intestinal sections with staining for ZO-1 and exposure-matched images with staining for occludin. Injury caused increase in the paracellular space between adjacent epithelial cells (**arrow**) stained for ZO-1. Nicotine prevented burn-induced decrease in occludin expression and maintained the normal localization of occludin at areas of cell contact. Scale bar = 10 μm.

raises the question of the mechanism by which the anti-inflammatory signal travels from the vagus nerve to the macrophages of the spleen. A recent report by Vida et al[27] further defined this signaling pathway, showing that the vagus nerve transmits its anti-inflammatory signal to the splenic nerve via α-7 nAchR, which then modulates splenic inflammatory cells.

These findings suggest that VNS may modulate inflammation via α-7 nAchR located on neurons. A similar mechanism may play a role in the ability of the vagus nerve to protect against gut barrier failure by signaling through vagus nerve connections to the enteric nervous system. Activation of NF-κB in intestinal epithelial cells alters tight junction protein expression, resulting in intestinal barrier failure.[28] Animals that have undergone vagotomy before intestinal injury after exposure to dextran sulfate sodium exhibit increased intestinal NF-κB activity, compared with animals with an intact vagus nerve.[29] Preventing nuclear translocation of NF-κB has been shown to improve barrier integrity and tight junction pro-

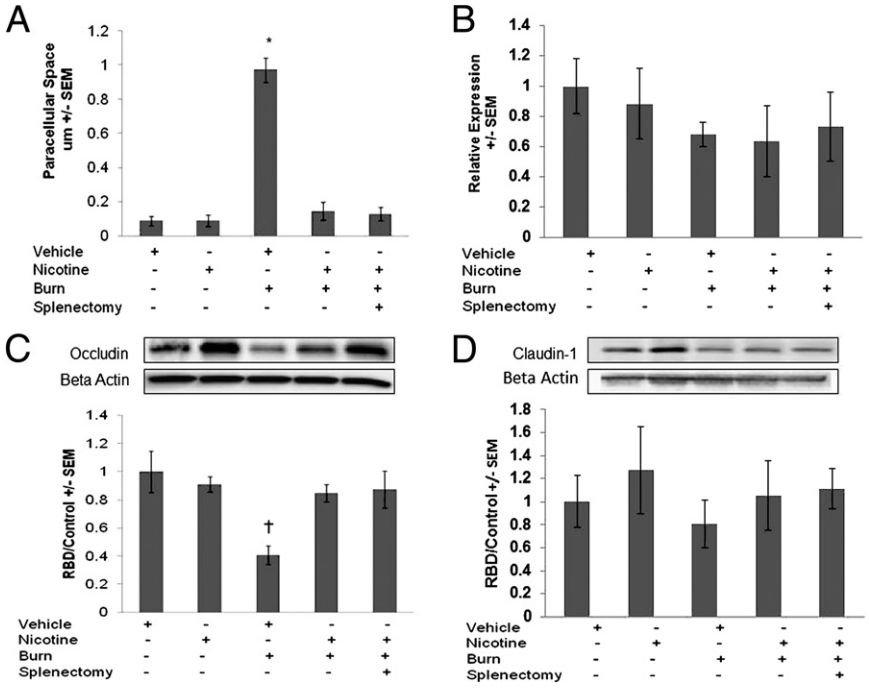

Figure 5. Nicotine modulates intestinal tight junction protein expression after injury. **A:** Quantification of the paracellular space between adjacent epithelial cells stained for ZO-1. Nicotine prevents the burn-induced increase in paracellular space between adjacent cells. **B:** Although nicotine prevented altered localization of ZO-1, there was no difference in ZO-1 gene expression at 4 hours after injury. **C** and **D:** Representative bands from Western blots for expression of intestinal occludin (**C**) and claudin-1 (**D**). Quantification of all band densities obtained showed that nicotine prevents injury-induced loss of intestinal occludin. There was no difference in intestinal claudin-1 expression between experimental groups. Western blot data are presented as relative band density (RBD), compared with control. $^{*}P < 0.001$ versus burn or vehicle; $^{\dagger}P < 0.05$ versus all other groups.

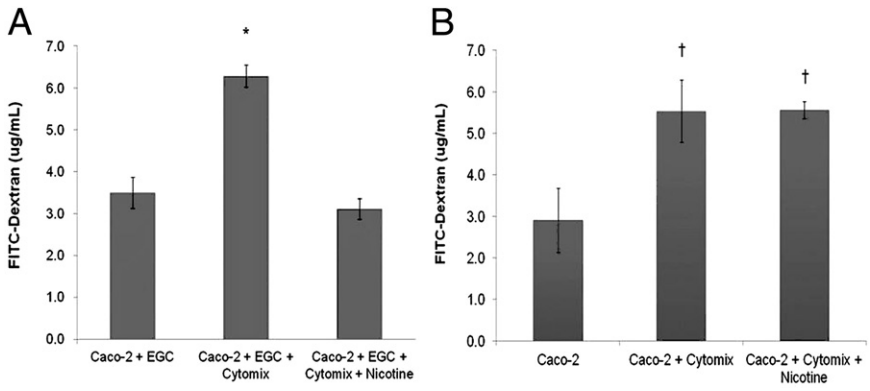

Figure 6. Nicotine improves *in vitro* epithelial barrier function after an inflammatory insult. Caco-2 cells were cultured alone or cocultured with enteric glial cells (EGCs) before stimulation with proinflammatory cytokines using Cytomix. *In vitro* permeability to 4-kDa FITC-dextran was measured to assess barrier function. **A:** The addition of nicotine to Caco-2/EGC coculture prevented the Cytomix-induced increase in epithelial permeability. **B:** Caco-2 cell monolayers cultured in the absence of EGCs. Nicotine demonstrated no protective effect on epithelial barrier function after stimulation with Cytomix, suggesting that the barrier-protective effects of nicotine require EGCs. $^{*}P < 0.05$ versus all other groups; $^{\dagger}P < 0.05$ versus Caco-2 cells alone.

tein expression in both *in vitro* and *in vivo* models, and it may play a role in the barrier-protective effects of VNS.[30,31] Stimulation of the vagus nerve has also been shown to alter inflammatory signaling in peritoneal macrophages by activating tyrosine protein Janus kinase 2 (JAK-2) and the anti-inflammatory effects of the transcription factor STAT3, which resulted in decreased intestinal inflammation and reduced postoperative ileus.[32] These effects were reproduced by treatment with nicotine, further supporting the importance of α-7 nAchR in mediating the inflammatory response of the gastrointestinal tract.

We have previously shown that VNS prevents gut barrier failure in models of severe burn injury[21] and traumatic brain injury.[33] Although VNS limits systemic inflammation by modulating systemic cytokine production by splenic macrophages, our studies suggest that the gut-protective effects of VNS are independent of the spleen, because those beneficial effects on the intestinal mucosa and tight junction protein expression are preserved in splenectomized animals.[18] In the present study, we investigated whether α-7 nAchR in the gastrointestinal tract may modulate gut protection after VNS. We documented the presence of α-7 nAchR on cultured intestinal epithelial cells and enteric glial cells. In addition, we also confirmed its presence in the gastrointestinal tract of animals used in our model of severe burn injury. As expected, there was no difference in expression of α-7 nAchR between sham-treatment and burn-injury animals. The expression of α-7 nAchR has been studied in other cell types, with receptor levels unchanged after inflammatory insults; however, receptor activation is known to cause changes in downstream immunomodulatory signaling.[34,35]

Nicotine is a cholinergic agonist known to decrease systemic inflammation after injury.[36,37] It does so by modulating inflammatory signals through α-7 nAchR, which is expressed on neurons, microglia, and inflammatory cells.[38,39] Nicotine has previously been shown to improve barrier function and tight junction protein expression in cultured intestinal epithelial cells.[40] Studies by Ghia et al[41,42] showed that the vagus nerve decreases gut inflammation in an animal model of colitis.[41] These anti-inflammatory effects were lost in vagotomized animals, but treatment with nicotine was able to restore the protective effects of VNS in vagotomized animals.[42] Both VNS and activation of α-7 nAchR with either nicotine or a specific pharmacological receptor agonist have also been shown to decrease postoperative ileus in an animal model, by inhibiting the inflammatory response of macrophages within the muscular layer of the small intestine.[32,43] These findings further suggest a link between cholinergic signaling and VNS-induced gut protection.

With our present series of experiments, we show that treatment with nicotine after severe burn injury prevents intestinal barrier breakdown and limits evidence of histological gut injury. The dose of nicotine given in these *in vivo* studies was based on several previous studies of the immunomodulatory effects of nicotine.[13,14] The present results are similar to the protective effects seen in injured animals who underwent VNS in our previous studies.[18,21,22] We also demonstrated that the improved gut barrier integrity is associated with improved expression and localization of the tight junction proteins occludin and ZO-1. The protective effects of nicotine administration were maintained in splenectomized animals, suggesting that gut protection in this model is independent of changes in the splenic inflammatory response. Nicotine does exert other anti-inflammatory effects, which may alter intestinal inflammation in this model through pathways that do not involve α-7 nAchR.[44] Other subunits of nAchR are present in the gut, and nicotine may bind these receptors and alter gut barrier function after injury.[45] Nicotine can also decrease cytokine production from inflammatory cells via noncholinergic receptors, which may alter the inflammatory state of the peritoneal cavity, and affect barrier function in this model.[46] Nicotine also modulates the inflammatory response of the central nervous system, and may alter signaling to the enteric nervous system through pathways that do not involve α-7 nAchR.[47]

The integrity of the gut barrier is an essential defense mechanism against sepsis and the development of multiple organ failure.[48,49] Intestinal barrier function and paracellular permeability are determined primarily by epithelial tight junction proteins.[50] Research in our laboratory has characterized the changes in expression and localization of these tight junction proteins after severe burn injury.[4,18,21,51–53] Occludin is an important structural protein, in that it attaches adjacent epithelial cells, forming a barrier within the paracellular space at the apical portion of the cell.[54] ZO-1 is an important component of the tight junction, in that it attaches occludin to the actin cytoskeleton within the intestinal epithelial cell.[55] Although we did not detect a change in ZO-1 expression in

the present study, we did identify changes in ZO-1 localization after injury, and these changes were modulated by treatment with nicotine. This finding is in agreement with previous studies showing that altered localization of ZO-1 alone is associated with increased barrier permeability.[56] Decreased expression and localization of occludin away from areas of cell-to-cell contact is also associated with barrier failure and increased paracellular permeability.[57,58] Alterations in occludin and ZO-1 in the gut have been observed after inflammatory insults, including sepsis, inflammatory bowel disease, necrotizing enterocolitis, and severe injury.[4,59–61]

The enteric nervous system regulates and controls functions of the gastrointestinal tract through sympathetic and parasympathetic pathways regulated by the central nervous system.[15,16,62] The mechanism by which the anti-inflammatory signal is transmitted from the vagus nerve to the intestinal barrier has yet to be defined. α-7 nAchR is known to transmit signaling from the vagus nerve to inflammatory cells of the spleen via the splenic nerve.[27] α-7 nAchR alters inflammatory signaling through its role as a perisynaptic receptor on neurons and through its expression on microglial cells. In the present study, we investigated whether α-7 nAchR may participate in signaling from the vagus nerve to the enteric nervous system. We documented the presence of α-7 nAchR in gut tissue and used nicotine, a cholinergic agonist, to prevent injury-induced gut barrier breakdown; our findings were similar to the protective effects we have previously shown after direct VNS. We used an *in vitro* model of intestinal epithelial cell and EGC coculture to further define the ability of nicotine to improve barrier function, which we had demonstrated in our animal model of severe burn injury. The barrier-protective effects of nicotine were lost in Cytomix-stimulated intestinal epithelial cells when EGCs were removed from the culture, suggesting that the barrier-protective effects of nicotine may be due to activation of EGCs. Future studies to further define the interactions among the vagus nerve, EGCs, and the intestinal epithelium may lead to development of targeted therapeutics aimed at reducing gut barrier failure and intestinal inflammation after severe injury.

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