Published in final edited form as:

J Trauma. 2011 May ; 70(5): 1168–1176. doi:10.1097/TA.0b013e318216f754.

Postinjury Vagal Nerve Stimulation Protects Against Intestinal Epithelial Barrier Breakdown

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Abstract

Background—Vagal nerve stimulation (VNS) can have a marked anti-inflammatory effect. We have previously shown that preinjury VNS prevented intestinal barrier breakdown and preserved epithelial tight junction protein expression. However, a pretreatment model has little clinical relevance for the care of the trauma patient. Therefore, we postulated that VNS conducted postinjury would also have a similar protective effect on maintaining gut epithelial barrier integrity.

Methods—Male balb/c mice were subjected to a 30% total body surface area, full-thickness steam burn followed by right cervical VNS at 15, 30, 60, 90, 120, and 150 minutes postinjury. Intestinal barrier dysfunction was quantified by permeability to 4 kDa fluorescein isothiocyanate-Dextran, histologic evaluation, gut tumor necrosis factor- α (TNF- α) enzyme-linked immunosorbent assay, and expression of tight junction proteins (myosin light chain kinase, occludin, and ZO-1) using immunoblot and immunofluorescence.

Results—Histologic examination documented intestinal villi appearance similar to sham if cervical VNS was performed within 90 minutes of burn insult. VNS done after injury decreased intestinal permeability to fluorescein isothiocyanate-Dextran when VNS was 90 minutes after injury. Burn injury caused a marked increase in intestinal TNF- α levels. VNS-treated animals had TNF- α levels similar to sham when VNS was performed within 90 minutes of injury. Tight junction protein expression was maintained at near sham values if VNS was performed within 90 minutes of burn, whereas expression was significantly altered in burn.

Conclusion—Postinjury VNS prevents gut epithelial breakdown when performed within 90 minutes of thermal injury. This could represent a therapeutic window and clinically relevant strategy to prevent systemic inflammatory response distant organ injury after trauma.

Keywords

Vagus nerve stimulation; Gut barrier; Burn; Tight junction

The intestinal epithelial barrier is a necessary and dynamic semipermeable structure that is charged with preventing normal intestinal flora, residing in a hostile environment, from causing catastrophic injury to their host. The intestinal tight junction is the last line of defense against the resident bacteria within the intestinal lumen.¹ The breakdown of the intestinal barrier could be the gateway to multiple organ failure as seen in sepsis, trauma, and hemorrhagic shock.² Despite the importance of the epithelial barrier, it is not immune to injury. The breakdown of the epithelial barrier has been shown to occur in various disease models including burn, traumatic brain injury, necrotizing enterocolitis, and Crohn's disease.^{3–6}

The tight junction itself is a complex structure that undergoes dynamic changes in response to injury. We have previously shown that burn insult changes the expression of the tight junction proteins occludin and ZO-1.³ The breakdown of the epithelial barrier and changes of tight junction protein expression in response to inflammation are due, in part, to the activation of myosin light chain kinase (MLCK), phosphorylation of myosin II light chain, and contraction of the actin-myosin ring.^{7–9} Our research has shown that in response to burn injury, a significant inflammatory reaction in the gut upregulates MLCK and increases phosphorylation of myosin II light chain, leading to tight junction separation.¹⁰

The potent anti-inflammatory effect of efferent vagal nerve stimulation (VNS), the cholinergic anti-inflammatory pathway, has been shown to play a significant role in prevention of an overwhelming inflammatory response.¹¹ Following this research, however, much has been focused on the specific stimulation of the $\alpha 7$ nAChR on macrophages and citing the spleen as the major contributing source of tumor necrosis factor (TNF)- α contributing to systemic inflammation.^{12,13} We have recently demonstrated an expanded anti-inflammatory role of the parasympathetic nervous system through efferent vagus nerve stimulation. Our research has shown that severe burn injury produces a secondary, indirect injury to the gut that is independent of splenic influence, yet responsive to anti-inflammatory stimulus provided by vagus nerve stimulation.¹⁴

We have also shown that vagus nerve stimulation before thermal injury has protective effects against gut epithelial barrier breakdown.¹⁵ We hypothesized that VNS performed after the initial burn insult would similarly protect the intestinal epithelial barrier and maintain its integrity. Additionally, could a therapeutic window be established for employment of vagal nerve stimulation?

MATERIALS AND METHODS

Thermal Injury Model

Male balb/c mice weighing 24 g to 28 g were obtained from Jackson Laboratories (Sacramento, CA). Animals were anesthetized with inhaled isoflurane before the experimental protocol. Animals underwent dorsal fur clipping with an electric clipper before

30% total body surface area (TBSA) dorsal steam burn for 7 seconds using a template designed to estimate 30% TBSA.¹⁶ After burn, animals received a subcutaneous injection of normal saline containing buprenorphine in a nonburned area for fluid resuscitation and pain control. Animals were recovered from anesthesia and returned to their cages where they were provided food and water ad libitum. All animal experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee.

Vagal Nerve Stimulation

A right cervical neck incision was performed at 15, 30, 60, 90, 120, or 150 minutes after thermal insult, and the right cervical vagus nerve exposed. Stimulation of the right cervical vagus nerve was performed using a VariStim III probe (Medtronic Xomed, Jacksonville, FL) at 2 mA, intermittently for 10 minutes. After nerve stimulation, the incision was closed with interrupted silk suture. Sham animals underwent right cervical incision and exposure of the vagus nerve but did not receive electrical stimulation.

Tissue Harvest

Animals were killed 4 hours after injury and tissues harvested. Segments of distal small intestine were removed and immediately snap frozen in liquid nitrogen before storage at -80°C for analysis. Segments of distal small intestine were also harvested and fixed in 10% buffered formalin (Richard Allan Scientific, Pittsburgh, PA) or optimal cutting technique (OCT) embedding media (Sakura Finetek, Torrance, CA) for histologic evaluation.

Histologic Evaluation

Segments of distal ileum were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Hematoxylin and eosin staining of the intestine was performed by University of California San Diego Histology Core Services. Sections were viewed via light microscopy and reviewed by a pathologist, who was blinded to the experimental groups. Three randomly selected fields from each specimen were graded based on a scoring system characterizing gut injury on a scale from 0 to 4: 0 = normal, no damage; 1 = mild, focal epithelial edema; 2 = moderate, diffuse swelling, necrosis of the villi; 3 = severe, diffuse pathology of the villi with evidence of neutrophil infiltration in the submucosa; 4 = major, widespread injury with massive neutrophil infiltration and hemorrhage as previously described.⁴

Intestinal Permeability Assay

An in vivo intestinal permeability assay was performed to assess intestinal barrier function (n = 4 animals per group). Four hours after injury, animals were anesthetized with inhaled isoflurane. A midline laparotomy incision was performed, and a 5-cm segment of distal ileum was isolated between silk ties. A solution of 200 μL containing 4 kDa fluorescein isothiocyanate (FITC)-Dextran (25 mg/mL; Sigma, St. Louis, MO) diluted in phosphate-buffered saline (PBS) was injected into the lumen of the isolated segment of intestine. The bowel was returned to the abdominal cavity and the abdomen closed. The animal was maintained lightly under general anesthesia for 30 minutes, at which time systemic blood was drawn by cardiac puncture and placed in heparinized Eppendorf tubes on ice. Plasma was obtained by centrifuging the blood at 10,000g for 10 minutes at -4°C . Plasma

fluorescence was measured in a fluorescence spectrophotometer (SpectraMax M5, Molecular Devices, Sunnyvale, CA) and compared with a standard curve of known concentrations of FITC-Dextran diluted in mouse plasma.

Enzyme-Linked Immunosorbent Assay

TNF- α was measured from gut tissue extracts of distal ileum harvested at 4 hours after injury. A commercially available enzyme-linked immunosorbent assay kit was obtained and used from R&D Systems (Minneapolis, MN). TNF- α was measured in picograms per milligrams tissue protein.

Occludin and MLCK Expression

Distal small intestine harvested from animals at 4 hours after burn (n = 4 animals per group) were homogenized in ice-cold tissue protein extraction reagent containing 1% protease, 1% ethylenediaminetetraacetic acid, and 1% phosphatase inhibitors (Pierce Biotechnology, Rockford, IL). The homogenized tissue was centrifuged at 10,000g for 5 minutes, the supernatants collected, and the protein concentration of each sample was measured using the bicinchoninic protein assay (Pierce Biotechnology). Protein was suspended in sodium dodecyl sulfate sample buffer and boiled for 5 minutes. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 8% to 16% Trisglycine gradient gels (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked with 5% bovine serum albumin (BSA; Sigma) in Tris- buffered saline /Tween 20 for 1 hour. Membranes were then incubated overnight at 4°C in primary antibody for occludin (Zymed, Carlsbad, CA) or MLCK (Sigma) prepared in a 1:500 concentration in 5% BSA/Tween 20. The membranes were then washed and incubated with a horseradish peroxidase-linked anti-rabbit IgG secondary antibody (Cell Signaling) before application of the Pierce Supersignal West Pico Chemiluminescent Kit for antibody detection. Luminescence was detected using the Xenogen IVIS Lumina (Caliper Life Science, Hopkinton, MA) imaging system. Mean pixel density of each sample was estimated using UN-SCAN-IT Gel Digitizing software (Silk Scientific, Orem, UT). The relative band density of each band was calculated by dividing the pixel density of each sample by the mean pixel density of the sham samples.

Confocal Microscopy

Segments of distal ileum (n = 3 animals per group) were embedded in OCT media and stored at -80°C or paraffin. OCT sections of intestine were cut 10- μ m thick using a Reichert-Jung Cryocut 1800 at -20°C (Reichert Microscopes, Depew, NY). OCT sections were fixed onto glass slides with 3.7% paraformaldehyde (Electron Microscopy Series, Hatfield, PA) for 10 minutes, washed with PBS, and then permeabilized with 0.01% Triton X-100 (Sigma) for 1 minute. Paraffin sections were rehydrated and incubated in citrate buffer (Thermo Scientific, Fremont, CA) for 20 minutes for antigen retrieval. Sections were washed once again in PBS before blocking for 1 hour in 3% BSA (Sigma). The sections were then incubated overnight in the occludin or ZO-1 (Zymed) antibody. Next, they were treated with Alexa Fluor 488 (Invitrogen) goat anti-rabbit antibody secondary in 1% BSA for 1 hour. Prolong Fade (Invitrogen) was added upon placement of cover slips. Images

were viewed using the Olympus Fluoview laser scanning confocal microscope with exposure-matched settings (Advanced Software v1.6, Olympus) at 60× magnification.

Statistical Analysis

Data are expressed as the mean $\pm$ the SEM. The statistical significance among groups was determined using analysis of variance with Student-Newman-Keuls correction where appropriate. Statistical significance for gut injury scoring was performed using the Kruskal-Wallis test for nonparametric data with post hoc Mann-Whitney test performed in pair wise fashion. Statistical analysis was performed using KaleidaGraph Software version 4.04 (Synergy Software, Reading, PA). Statistical significance was defined as $p < 0.05$.

RESULTS

Postinjury VNS Attenuates Intestinal Injury

Sections of distal ileum from sham, burn, and VNS-treated animals 4 hours after 30% TBSA burn underwent histologic evaluation to confirm the presence of intestinal injury. Figure 1, A demonstrates the normal appearance of intestinal villi in the sham animal. The distal small intestine from burned animals displayed histologic pathology characterized by blunting of the villi (Fig. 1, B). The protective effects of VNS in the postinjury model were seen as long as the VNS is performed within 90 minutes of injury (Fig. 1, C–F). Performing VNS at 120 minutes and 150 minutes postinjury resulted in intestinal appearance similar to burn alone (Fig. 1, G and H). Gut injury scores were significantly lower in groups that received VNS within 90 minutes of injury. Scores were similar to burn alone when VNS was performed at 120 minutes and 150 minutes after thermal insult.

Postinjury VNS Prevents Intestinal Permeability

Intestinal barrier integrity was tested in an in vivo assay using small molecular weight FITC-Dextran (Fig. 2). Intestinal permeability was increased in burned animals consistent with previous experiments.¹⁵ Postinjury VNS produced the same pattern of protection as seen in histology. Performed within 90 minutes of injury, VNS protected against the breakdown of the gut epithelial barrier and intestinal permeability was similar to sham. Protection offered to burns was lost if performed at 120 minutes or later following thermal insult, and permeability was similar to burn animals.

Postinjury VNS Reduces Intestinal Inflammation

To assess intestinal inflammation, TNF- α was measured by enzyme-linked immunosorbent assay in gut tissue at 4 hours after thermal insult and in sham animals (Fig. 3, A). Burn induced a significant increase in intestinal TNF- α compared with sham. Postinjury VNS significantly reduced intestinal inflammation compared with burn alone if performed within 90 minutes of injury. Intestinal TNF- α levels began to increase again starting at 90 minutes and became significantly elevated compared with sham animals at 150 minutes. This demonstrates that VNS continued to have anti-inflammatory effects on intestinal TNF- α production 2 hours after burn insult.

MLCK is an important epithelial tight junction protein that is increased in response to inflammation. We performed Western blot to determine MLCK levels 4 hours after 30% TBSA burn in the distal ileum (Fig. 3, B). The expression pattern mirrored TNF- α levels. Burn insult resulted in a significant increase of MLCK levels compared with sham. Animals that had VNS performed after 120 minutes also demonstrated a significant increase in MLCK levels.

Postinjury VNS Maintains Intestinal Tight Junction Integrity

Immunofluorescence of the intestinal tight junction protein ZO-1 was performed and visualized using confocal microscopy. Figure 4, A–I show the tight junction appearance of ZO-1 in sham animals. The epithelial cells of the distal ileum are seen as confluent outlines around the cells. Burn injury caused large gaps between cells to appear as seen at the arrows in Figure 4, B and J. Postinjury VNS performed within 60 minutes of injury maintained the integrity of the intestinal tight junctions evidenced by the lack of gaps between cells as seen in Figure 4, C–E and K–M. At 90 minutes postinjury, VNS did preserve a majority of the tight junctions similar to sham (Fig. 4, F and N). However, cells started to separate from each other (arrowhead). VNS performed at 120 minutes and 150 minutes after burn injury shows a similar gap pattern to that seen with burn alone (Fig. 4, G, H, and O, P).

Immunofluorescence of a second tight junction protein, occludin, was also performed and visualized using confocal microscopy. The exposure-matched images in this series were quantitative giving an overall impression of the quantity of occludin expressed. Figure 5, A–I show the normal, robust staining pattern for occludin at the cell periphery within intestinal villi. After burn injury, the expression of occludin is not only degraded, but also the normal peripheral staining pattern around the outer border of individual cells is lost (Fig. 5, B and J). Postinjury VNS maintains the normal expression pattern and quantity if performed within 60 minutes of injury (Fig. 5, C–E and K–M). VNS performed 90 minutes after thermal insult largely maintains the expression pattern of occludin (Fig. 5, F and N); however, a breakdown and degradation of the staining are apparent (arrowhead). VNS performed after 120 minutes or 150 minutes demonstrated a similar staining pattern to burn animals (Fig. 5, G, H and O, P).

Western blot analysis of occludin confirmed what was seen with immunofluorescence via confocal microscopy (Fig. 5, Q). Burn injury resulted in significant reduction in intestinal occludin expression. VNS performed within 60 minutes of insult maintained occludin expression similar to sham. However, after 90 minutes, occludin expression was elevated but no longer significantly elevated compared with sham. The lack of a statistically significant difference at 90 minutes after injury and sham indicates that there likely is an effect of VNS on occludin expression. This effect may be occurring too late to overcome the degradation induced by injury.

DISCUSSION

The importance of the vagus nerve and the use of parasympathetic stimulation as an anti-inflammatory strategy have been well demonstrated. However, little information is available as to the extent of protective effects offered by efferent VNS in a postinjury model. In this

series of experiments, we investigated the protective effects of VNS on the gut mucosal barrier in a postthermal insult model. We establish here that there is a significant therapeutic benefit to VNS performed within 90 minutes of injury. Not only does this demonstrate a “therapeutic window” for intervention, but it also answers the question as to whether VNS is preventing barrier breakdown or speeding barrier recovery. The results show clearly that there is consistency in appearance, permeability, and protein expression when VNS is performed within 90 minutes of burn injury. These data demonstrate that VNS is preventing gut mucosal barrier breakdown after burn insult.

The parasympathetic pathway has a potent anti-inflammatory influence on intestinal inflammation and injury.^{14,15} In this study, a reduction in inflammation was achieved with low levels of local TNF- α production from the gut, similar to sham, in VNS-treated animals. Further evidence for the anti-inflammatory effects in this model is the reduction in the activation of MLCK. Increases in MLCK are known to occur in response to activation of the nuclear factor- κ B pathway, of which TNF- α is a known potent activator.⁷

This is the first study, to our knowledge, to demonstrate a “therapeutic window” for mucosal barrier protection via VNS in a postinjury model. Previous studies have suggested that postinjury stimulation may have protective effects on hemodynamics, acute lung injury, and reduction in inflammatory markers.¹⁷ However, no study has applied this postinjury, preventive strategy to the gut mucosal barrier.

The importance of these results could have very significant, clinically relevant implications in the treatment of the trauma patient. For many years, investigators have postulated that the integrity of the gut epithelial barrier is paramount in prevention of postinjury sepsis.¹⁸ Gut barrier breakdown is consistent across models of trauma, hemorrhagic shock, burn, necrotizing enterocolitis, and Crohn’s disease. The effectiveness of vagus nerve stimulation at maintaining gut epithelial barrier integrity has now been demonstrated in a pre and postinjury model.¹⁵ This could signify a relevant strategy in the prevention of multiple organ failure in the clinical setting.

Studies have also suggested that VNS elicits its protective effects through the α 7nACh receptor located on the surface of splenic macrophages.^{12,13} Recent findings from our laboratory have suggested alternative, or perhaps additional, beneficial effects of vagus nerve stimulation. We have demonstrated that the protective effects of vagus nerve stimulation on the gut act independent of the spleen and may be mediated through the activation of enteric glia.¹⁴

In addition to the signaling occurring between vagus nerve and intestinal epithelial cell, another question arises. Why is the protective effect of VNS lost when not performed in a timely manner after injury? It is clear from this study that VNS loses its protective effect if performed after 90 minutes from injury. Within 90 minutes of injury, inflammation and pathology in the intestine is reduced, the integrity of the intestinal epithelial barrier breaks down, and expression of the tight junction proteins MLCK and occludin are altered. One explanation may be that the local inflammatory reaction generated within the gut is too

robust or too involved at this time point for VNS to overcome. There may also be an inflammatory threshold that once crossed, it cannot be reversed or blunted with VNS.

In summary, postinjury VNS preserves intestinal villous structure, prevents the breakdown of the intestinal epithelial barrier by anti-inflammatory effects, and prevents effects on tight junction protein expression. This study also demonstrates that there is a therapeutic window in which to perform the intervention. The postinjury model could translate into the clinical setting much more so than our previous preinjury models. If applicable in humans, this may represent a potent strategy for limiting posttraumatic, sterile inflammatory responses and improve outcomes after severe injury.

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DISCUSSION

Dr. Basil A. Pruitt, Jr. (San Antonio, Texas): Dr. Krzyzaniak, you and your colleagues have added a new chapter to the fine work from Dr. Coimbra's lab which has previously shown a relationship between a post-injury increase in myosin light chain kinase (MLCK) and loss of intestinal epithelial barrier function associated with loss of tight junction integrity. These new studies indicate that vagal nerve stimulation applied up to 90 minutes post-burn ameliorates the intestinal injury in a murine model of a 30 percent burn.

There are a few questions that the authors need to address to help us evaluate their findings.

1. Since the interval between vagal stimulation and tissue harvest ranged from 215 minutes to only 80 minutes, do the findings of lesser effect with later application of vagal stimulation simply mean that insufficient time had elapsed after vagal stimulation for the effects to be evident in those animals stimulated at 90 minutes and beyond?
2. Could a vagal effect on intestinal blood flow be at least in part responsible for these findings?
3. Are the effects of vagal stimulation affected by the severity of injury? That is, is longer stimulation required to achieve the same results in animals with a 60 percent burn?
4. Do the effects of vagal stimulation persist beyond four hours or is repeated or even continuous stimulation required to maintain intestinal integrity?
5. Since changes in intestinal permeability have been related to bacterial translocation, do you have correlative intestinal lymph node bacterial counts?
6. Do you think that vagal stimulation would be protective in a non-burn ischemia reperfusion injury of the intestine?

And, lastly, does anyone who has had a total vagotomy have to seek other treatment to obtain the same beneficial effect?

Dr. Lawrence N. Diebel (Detroit, Michigan): I can't beat Dr. Pruitt. I have one – I had the question about the blood flow changes, too, so I have that same question. But the other question I have is the following.

Parasympathetic agonists have been shown to increase the receptor for IGA in intestinal epithelial cells and you need that receptor to transport IGA and I wonder if they have any – and since IGA is so important for barrier function do they have any data by doing

parasympathetic stimulation do you increase IGA levels in the gut and that's why it's protective for all these adverse effects you saw?

Dr. Michael J. Krzyzaniak (San Diego, California): Thank you very much Dr. Pruitt for your comments and questions. Regarding the first question, I don't think it is a matter of insufficient time elapsed between stimulation at later time points and length of time needed to see an effect. Rather, it's more likely that the amount of inflammation generated from the burn stimulus is too extensive to overcome with vagal stimulation after 90 minutes.

In response to the second question regarding the effect of vagal stimulation on intestinal blood flow, we certainly know that intestinal ischemia/ reperfusion causes significant injury. It is possible that the vagal stimulation is improving intestinal blood flow, perhaps by blocking or blunting the sympathetic vasoconstriction associated with the injury response.

I think your third question about the effects of vagal nerve stimulation being affected by the severity of injury is very interesting. We have maintained the 30% TBSA model because of the consistent injury pattern it provides. As we move forward with drug candidate experiments acting in lieu of vagus nerve stimulation, it would be very interesting to look at more severe injury models.

To answer your fourth question, we have seen that the injury pattern to the intestine is maximal at the 4–6 hour mark and virtually restored to normal by 24 hours. This finding would suggest that there is a therapeutic window early in the course of injury, since the gut appears to restore itself relatively quickly. We have looked at effects on lung injury 24 hours after the burn and that data would suggest that either the protective effect is truly persisting beyond 4 hours or the development of acute lung injury is based on a gut-lung axis where injury to one leads to secondary injury of the other.

And then regarding your question about intestinal lymph node sampling and bacterial translocation, we have begun to investigate these conditions and their effects on mesenteric lymph in our laboratory in a larger animal model. This is an excellent question and we're looking forward to seeing what the data shows.

To answer your next question, I do think vagal stimulation will be protective in a non-burn ischemia/reperfusion model. We are currently expanding our models to include trauma/ hemorrhagic shock (T/HS) and mesenteric artery occlusion. Thus far, in the T/HS model, we have seen intestinal protection with vagal stimulation, and the data are still being compiled for the mesenteric occlusion model.

And finally, Dr. Pruitt, even though you have had a total vagotomy, I do not think you need to seek another treatment at this time. However, stay tuned, we'll let you know once we start clinical trials.

Dr. Diebel, we have not looked specifically at effects of IGA or quantifying IGA within the gut. That is an interesting question, however our focus has not been on effects on local intestinal antibody levels.

Figure 1.

Gut histology and gut injury scoring. VNS preserves intestinal architecture in the postinjury model. Sections of distal ileum harvested 4 hours after 30% TBSA burn (n = 3 animals per group) were stained with hematoxylin and eosin. All images are taken at 20× magnification. (A) Representative image from sham animal showing normal intestinal villi architecture. (B) Representative image from an animal receiving 30% TBSA burn without VNS demonstrating shortened intestinal villi with blunted tips. (C–F) Animals treated with VNS after burn at 15, 30, 60, and 90 minutes, respectively. These images show a preservation of normal intestinal villous anatomy. (G, H) Sections from animals treated with VNS 120 minutes and 150 minutes after 30% TBSA display pathology similar to what is seen in burn alone. Black bar = 100 μ m. (I) Intestinal injury was scored on a scale of (0) normal to (4)

severe by a pathologist blinded to the experimental protocol. Gut injury scores were significantly elevated in the Burn, Burn/stm@120, and Burn/stm@150 groups ($p < 0.05$) using Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney test for pair wise comparison.

Figure 2.

Permeability to 4 kDa FITC-Dextran. Postinjury VNS limits intestinal barrier dysfunction after 30% TBSA burn (n = 4 animals per group). Treating animals with VNS significantly reduces intestinal permeability if performed within 90 minutes of injury. Permeability increases to levels similar to burn if VNS is performed 120 and 150 minutes after injury. Data are expressed as mean systemic FITC-Dextran concentration in $\mu\text{g/mL} \pm \text{SEM}$. * $p < 0.05$ versus Burn; # $p < 0.05$ versus Burn/stm@120; and + $p < 0.05$ versus Burn/stm@150 using analysis of variance.

Figure 3.

Intestinal TNF- α and MLCK levels. Intestinal inflammation is reduced by postinjury VNS.

(A) Intestinal TNF- α levels measured from segments of distal ileum 4 hours after 30% burn using ELISA (n = 4 animals per group). VNS prevents the increase in TNF- α seen after burn when performed within 90 minutes injury. Inflammation evidenced by a rise in TNF- α levels begins to increase in groups stimulated after 120 minutes. Data are expressed as picograms per milligrams of gut tissue $\pm$ SEM. $*p < 0.05$ versus Burn and $\#p < 0.05$ versus Burn/stm@150 using analysis of variance. (B) Changes in intestinal MLCK expression 4 hours after burn insult were measured using Western Blot. Burn induces a significant rise in MLCK expression over sham. Postinjury VNS abrogates the rise in MLCK expression when performed within 90 minutes of injury. $*p < 0.05$ versus Burn; $\#p < 0.05$ versus Burn/stm@120; and $+p < 0.05$ versus Burn/stm@150 using analysis of variance.

Figure 4.

ZO-1 tight junction protein immunofluorescence. VNS maintains the integrity of the tight junction between intestinal epithelial cells. The cell-to-cell proximity is demonstrated by ZO-1 immune staining (green), viewed with confocal microscopy. All images are pictured under 60 $\times$ magnification. The normal close association of the tight junction is shown in sham animal (Panel A, I). Burn injury causes a dissociation of the normal tight junction with separation between cells, highlighted by the arrows in Panel (B & J). VNS performed within 60 minutes following injury universally maintains the intestinal tight junction (Panel C, D,

E, & K, L, M). VNS performed within 90 minutes post-injury largely maintains the close cell-to-cell association (Panel F & N). However, when the image is zoomed in, there is evidence of tight junction stress seen at the arrowhead with small amounts of separation between cells. VNS performed 120 minutes after injury, and more pronounced at 150, shows significant tight junction separation similar to burn animals (Panel G, H & O, P). White bar = 20 μ m.

Figure 5.

Occludin immunofluorescence and Western Blot. VNS maintains the expression levels of the intestinal tight junction protein, Occludin, seen with immunofluorescence (green) if performed within 90 minutes of thermal injury. Images are exposure-matched to highlight quantity of expression and taken at 60× magnification with adjacent zoomed in images. Normal staining appearance is seen in Panel (A, I) from sham animal with intense staining at the periphery of the cell. Burn insult (Panel B, J) causes disorganization in the staining pattern. Because the images are color-matched, the lower intensity of staining signifies

lesser protein expression in the tissue. VNS performed within 60 minutes after injury maintains the normal architecture and intensity of staining (Panel C, D, E & K, L, M). VNS 90 minutes after injury does maintain the architecture (Panel F). However, there is some evidence that protein expression may be degraded as seen at the arrowhead (Panel N). VNS performed at 120 and 150 minutes after burn produces patterns and intensities similar to burned animals not receiving VNS (Panel G & O, H & P). White bar = 20 μ m. Occludin western blot coincided with what was seen with immunofluorescence (Panel Q). VNS performed within 60 minutes of thermal insult significantly increased intestinal occludin expression compared to burn. * $p < 0.05$ vs. Burn by analysis of variance. However, the alteration in occludin expression seen when VNS was performed after 90, 120, and 150 minutes, did not reach statistical significance.