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Efferent vagal nerve stimulation attenuates acute lung injury following burn: The importance of the gut-lung axis

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Abstract

Background—The purpose of this study was to assess acute lung injury when protection to the gut mucosal barrier offered by vagus nerve stimulation is eliminated by an abdominal vagotomy.

Methods—Male balb/c mice were subjected to 30% total body surface area steam burn with and without electrical stimulation to the right cervical vagus nerve. A cohort of animals were subjected to abdominal vagotomy. Lung histology, myeloperoxidase and ICAM-1 immune staining, myeloperoxidase enzymatic assay, and tissue KC levels were analyzed 24 hours after burn. Additionally, lung IκB-α, NF-κB immunoblots, and NF-κB-DNA binding measured by photon emission analysis using NF-κB-luc transgenic mice were performed.

Results—Six hours post burn, phosphorylation of both NF-κB p65 and IκB-α were observed. Increased photon emission signal was seen in the lungs of NF-κB-luc transgenic animals. Vagal nerve stimulation blunted NF-κB activation similar to sham animals whereas abdominal vagotomy eliminated the anti-inflammatory effect. After burn, MPO positive cells and ICAM-1 expression in the lung endothelium was increased, and lung histology demonstrated significant injury at 24 hours. Vagal nerve stimulation markedly decreased neutrophil infiltration as demonstrated by MPO immune staining and enzyme activity. Vagal stimulation also markedly attenuated acute lung injury at 24 hours. The protective effects of vagal nerve stimulation were reversed by performing an abdominal vagotomy.

Conclusion—Vagal nerve stimulation is an effective strategy to protect against acute lung injury following burn. Moreover, the protective effects of vagal nerve stimulation in the prevention of acute lung injury are eliminated by performing an abdominal vagotomy. These results establish the importance of the gut-lung axis after burn in the genesis of acute lung injury.

Pulmonary complications following burn injury have been well characterized for over 30 years.¹ Although recent studies suggest that the overall mortality rates for patients with acute lung injury (ALI) or the acute respiratory distress syndrome (ARDS) from all causes

may be decreasing,² ALI remains a significant cause of morbidity and mortality³ with an estimated 190,600 cases leading to 74,500 deaths per year in the US alone.⁴⁻⁶ Supportive care measures in the treatment of ALI/ARDS have evolved,⁷ but means of prevention are yet to be realized. Although these statistics represent all causes of acute lung injury, secondary pulmonary complications after burn remain pertinent. The initial stages of ALI involve large volumes of fluid influx into the lungs within the first 24 hours after burn.^{8,9} Consistent with these findings, we have used a mouse model of injury to demonstrate that ALI does occur after burn insult as early as 24 hours,¹⁰ which is also consistent with that seen with preclinical models of endotoxin mediated sepsis¹¹ and hemorrhagic shock.¹²

Pro-inflammatory mediator synthesis in response to infection, trauma, or burn often clinically leads to ALI/ARDS and Multiple Organ Dysfunction Syndrome (MODS). Tumor necrosis factor *alpha* (TNF- α), which is released in response to burn injury,¹³⁻¹⁵ is a proximal mediator for other inflammatory cytokines and chemokines that coordinate a cascade of interactions resulting in lung injury.^{16,17}

Activated neutrophils have been shown to be the effector cell type in acute lung injury, and the importance of activation of the NF- κ B pathway has also been well established in experimental models of injury.^{11,12,18-20} Following a thermal insult, TNF- α is released and the NF- κ B pathway is activated. NF- κ B activation leads to the transcription of inflammatory genes including IL-8, which, in turn, leads to neutrophil chemotaxis to specific sites of injury.^{13,19,20} In the pulmonary endothelium, ICAM-1 expression is upregulated in response to elevated TNF- α , facilitating the transendothelial migration of activated neutrophils into the lung interstitium, degranulation and release of enzymes, resulting in tissue damage.

Vagus nerve stimulation has been found to decrease the systemic inflammatory response syndrome (SIRS), to reduce systemic cytokine release, and to attenuate the systemic response to endotoxin.²¹ The “cholinergic anti-inflammatory pathway” acting on α -7 nicotinic acetylcholine receptors present on the surface of splenic macrophages has been shown to reduce systemic TNF- α levels.²² Moreover, when splenectomy is performed, the protective effect of VNS seems to be eliminated in an endotoxemia model.²³ Our laboratory has demonstrated that vagus nerve stimulation has a marked effect on the intestinal barrier after burn injury.²⁴ Prevention of the gut epithelial barrier breakdown would, therefore, deter translocation of bacterial products and eliminate activation of mesenteric lymph, possibly attenuating secondary distant organ injury.

Many have postulated that when the gut mucosal barrier breaks down, secondary end-organ dysfunction ensues. The gut has been shown to be a source of inflammatory cytokines with the capability of priming neutrophils^{25,26} and driving MODS.²⁷ Magnotti et al²⁸ have shown that the activation of mesenteric lymph contributes to distant organ injury in a burn model. We have recently demonstrated that burn injury specifically leads to local breakdown²⁹ of the intestinal barrier by inducing changes in the expression of tight junction proteins in the intestinal epithelial cells.^{24,29}

Recent studies have shown that targeted therapies to reduce SIRS associated with cutaneous burn have been effective in preventing distant organ injury in mice.^{30,31} We hypothesized

that by employing VNS, ALI after thermal insult would be minimized. We also postulated that if VNS protection to the intestine was eliminated via abdominal vagotomy, then ALI would occur as seen in burned mice without VNS treatment indicating that there is no direct protective effect of VNS on the lungs.

MATERIALS AND METHODS

Burn model of injury

Male balb/c mice (8–12 weeks, Jackson Laboratories, Sacramento, CA) were anesthetized using inhaled isoflurane. The dorsal fur was removed using an electric clipper. Animals were then placed in a template estimating 30% total body surface area (TBSA) and subjected to a steam burn for 7 seconds as previously described.^{14,32} Following burn injury, animals received a subcutaneous injection of 1.4 ml normal saline with 100 μ L of buprenorphine for pain control and resuscitation. The initial resuscitation volume is based on the Parkland Formula. Animals were recovered from anesthesia and returned to their cages. The animals were allowed ad libitum access to food and water until they were killed. At 6 and 24 hours following burn, animals were euthanized and tissue procurement was performed. Prior to tissue collection, intracardiac cold saline flush was performed via the right ventricle to clear the circulatory system of cellular components. Sham animals received dorsal shaving without resuscitative fluid administration or analgesia. Tissue collection from sham animals was performed in the same manner as the other groups. Experiments were approved by the University of California Animal Subjects Committee and are in accordance with guidelines established by the National Institutes for Health.

Vagal nerve stimulation

Following induction of general anesthesia with inhaled isoflurane and prior to thermal insult, a right cervical neck incision was performed and the right cervical vagus nerve was exposed. Vagal nerve stimulation (VNS) was performed using a VariStim III probe (Medtronic Xomed, Jacksonville, FL) with 2 mA current, at 1-second intervals, for 10 minutes prior to burn insult. The incision was closed with interrupted silk suture and the animal was immediately subjected to burn injury as previously described. Sham animals underwent right cervical incision and exposure of the vagus nerve but did not receive stimulation.

In one arm, surgical abdominal vagotomy (Vx) was performed immediately prior to vagal nerve stimulation and subsequent burn injury through an upper midline laparotomy incision. The gastro-esophageal junction was identified that the dorsal and ventral vagus nerve were visualized on the distal esophagus with an Olympus SZ61 stereomicroscope (Leeds Precision Instruments, Minneapolis, MN). Both branches of the vagus nerve were identified and divided sharply.

Histological evaluation

Lung samples taken 24 hours after burn injury were fixed immediately at the time of collection in 10% formalin solution and stored in paraffin. Hematoxylin & Eosin staining was performed by UCSD Histology Core Services. Multiple 5- μ m sections of lung ($n = 3$ mice per experimental condition) were evaluated by a board certified pathologist blinded to

the experimental groups. Sections were scored according to the pulmonary injury scoring system previously used by our laboratory.¹² Sections were rated on a scale from 0 to 3 ranging from normal to severe injury. Sections were analyzed based on amount of intra-alveolar hemorrhage, pulmonary congestion, edema, and infiltration of inflammatory cells to yield a maximum possible score of 12. Lung Injury Scores were averaged for each experimental condition and images were taken at 20× and 60× magnification with light microscopy.

Immunohistochemistry

Formalin fixed lung specimens were stored in paraffin by UCSD Histology Core Services. After deparaffinization, lung slides were incubated in citrate antigen retrieval solution (Thermo Scientific, Fremont, CA), for 20 minutes at 95°C and cooled to room temperature ($n = 5$ animals per group). Sections were blocked for 2 hours with 10% donkey serum albumin in PBS at room temperature. Next, slides were incubated overnight at 4°C with primary antibody for myeloperoxidase (Thermo Scientific) or ICAM-1 (R&D Systems, Minneapolis, MN) diluted 1:100 in 1% bovine serum albumin in PBS. Sections were washed and incubated in 0.3% H₂O₂ to quench endogenous peroxidase activity for 15 minutes. Sections were again washed and incubated in anti-rabbit IgG HRP-linked antibody (Cell Signaling Technologies, Danvers, MA) diluted 1:400 in 1% BSA in PBS for 1 hour. Specific labeling was done with DAB substrate kit (Vector Laboratories, Burlingame, CA). Specimens were then counterstained with hematoxylin. Direct comparison for ICAM-1 staining was performed with light microscopy. Ten random high power fields were examined per animal to determine the number of cells staining positive for MPO. Samples for both ICAM-1 and MPO immunostaining were prepared from tissue samples taken 24 hours after burn injury.

Myeloperoxidase assay

Frozen lung specimens taken from animals 24 hours after thermal injury were stored at -80°C until they were used to perform the enzymatic reaction. Approximately 5 mg of frozen lung tissue was dounce homogenized in 5% hexadecylammonium bromide solution, placed in a centrifuge at 14,000 rpm for 30 minutes at 4°C, and supernatant was removed. A standard curve was created using stock Myeloperoxidase enzyme 10 units/mL (Sigma, St. Louis, MO), reconstituted with 0.5 cc of ddH₂O. Samples were diluted 1:50 with 1× phosphate buffered saline solution. 10 microliters of stock standard curve dilutions and unknown samples were added to 90 μ L substrate solution comprised of 1:1 dilute hydrogen peroxide and 1 mg/mL DMB o-Dianisidine (Sigma) for a total volume of 100 μ L per well. Reaction was allowed to incubate at room temperature for 10 minutes. Absorbance was then measured on a plate reader at 450 nm. Data are expressed as units of MPO per mg of tissue lysate $\pm$ SEM.

Lung KC (IL-8) content

Mouse keratinocyte-derived chemoattractant (KC) is a neutrophil chemoattractant present in mice with functions that are similar to that of human IL-8. Quantification of KC was conducted through a commercially available enzyme linked immunosorbent assay (ELISA)

from R&D Systems. Samples taken from animals 6 hours after burn were stored at -80°C until they were dounce homogenized using a tissue protein extraction reagent (Thermo Scientific). Samples were diluted 1:5 in calibrator diluent provided in the kit. The total protein concentration of each extract was determined using the bicinchoninic acid (BCA) protein assay according to a microplate procedure (Pierce, Rockford, IL). Absorbance was measured at 562 nm with a standard curve for albumin. Data are represented as pg/mg of lung tissue protein.

Immunoblot

Lung samples taken from animals 6 hours after burn injury ($n = 5$ animals per group) were immediately snap frozen with liquid nitrogen at the time of collection and stored at -80°C . Tissue protein extraction was performed by dounce homogenizing tissue with Nuclear and Cytoplasmic Extraction Reagent (Pierce) and Halt Phosphatase and Protease Inhibitor (Pierce). Samples were then centrifuged and the supernatant was retained. The total protein concentration of each extract was determined using the BCA protein assay according to a microplate procedure (Pierce). Absorbance was measured at 562 nm with a standard curve for albumin. Ten micrograms of protein were loaded into each sample well. Samples were separated using 6–18% trisglycine gel and transferred onto nitrocellulose membranes. After blocking with 5% bovine serum albumin, samples were exposed overnight to rabbit anti-Phospho-I κ B- α , anti-Phospho-NF κ B p65 antibody (Cell Signaling Technology, 1:500). The samples were then treated with anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, 1:2000), followed by chemiluminescent detector solution (Pierce). Beta-actin and Lamin B loading control (Cell Signaling Technology, Danvers, MA; 1:500) were measured appropriately for each gel. Band pixel density was calculated using Un-Scan-It software (Silk Scientific, Orem, UT). The relative band density was calculated by dividing the pixel density of the lane of interest by its pixel density of the loading control. The average relative band density of the sham animals was calculated and the data is reported as Relative Band Density/Sham ($\pm$ SEM).

Detection of in vivo bioluminescence using transgenic NF- κ B-luc mice

Bioluminescence offers a very powerful technology yielding clear images produced using fresh tissues with minimal ischemic time. In these transgenic mice, when the NF- κ B binding element is activated by NF- κ B p65, the transcription of firefly luciferase ensues. When luciferase is exposed to its substrate, D-luciferin, light is emitted and quantification is possible by using a CCD camera. When the software records the photon output, color is used to express intensity of light emitted. Red colors signify more intense emission while violet colors signify the opposite. Transgenic mice with NF- κ B-responsive elements upstream of firefly luciferase (original male purchased from Jackson Labs) with strain name B10.Cg-H2^KTg(NF κ B/Fos-luc)26Rinc/J were raised in our laboratory with continuous availability of food and water. Animals ($n = 5$ animals per group) were subjected to 30% TBSA steam burn with or without VNS and abdominal vagotomy as outlined above. Six hours after burn (or dorsal shave for sham animals), animals were anesthetized with inhaled isoflurane and received 150 μL of D-luciferin substrate (15 mg/mL, Caliper Life Sciences, Hopkinton, MA) via intraperitoneal injection. Five minutes later, the lungs were harvested, rinsed in normal saline to remove excess blood, and subjected to a 5-minute exposure ex vivo under a deep-

cooled charge-coupled device (CCD) camera (IVIS Lumina Xenogen).³³ Living Image 3.1 software (Caliper Life Sciences) was used to quantify luminescent intensity using region of interest measurements of equivalent areas expressed as photons/sec/cm²/sr. All images were color matched on the same scale within the analyzing software. When images display no color, luminescence from that image may be present but does not register as it is beneath the minimum level of detection set within the software.

Statistical analysis

Data is expressed as the mean $\pm$ SEM. The statistical significance among groups was determined using analysis of variance with Bonferroni correction where appropriate. Nonparametric data were analyzed with Kruskal-Wallis test followed by Wilcoxon test for multiple comparisons. Statistical analysis was performed using KaleidaGraph Software version 4.04 (Synergy Software, Reading, PA). Statistical significance was defined as $P < .05$.

RESULTS

Histologic lung injury is ameliorated by VNS

Lung specimens (Fig 1) of burn animals (*B, F*) showed significant histologic changes, including edema, markedly increased inflammatory cell infiltration, intra-alveolar hemorrhage, and alveolar-capillary membrane thickening when compared to sham (*A, E*). In contrast, histologic assessment of lungs of animals that underwent VNS prior to burn injury (Panel *C, G*) showed similar appearance compared to sham. Performing abdominal vagotomy prior to VNS and burn eliminated the protective effect on the lungs (*D, H*) and produced images similar to burn alone.

The lung injury severity scores (Fig 2) were provided by a pathologist blinded to the study protocol, and were assessed 24 hours after burn insult for inflammatory cell infiltration, pulmonary edema, intra-alveolar hemorrhage, and pulmonary congestion. Using a board certified pathologist increases our confidence that the images demonstrated true injury due to burn as opposed to atelectasis. Lung injury scores were significantly higher in the burn group (8.5 ± 0.48) compared to either sham or VNS + burn (3.5 ± 0.81 and 2.3 ± 0.33 , respectively; $P < .05$). Abdominal vagotomy prior to VNS and burn abolished the protective effect of VNS with an average injury score of 8.5 ± 0.56 ($P < .05$ vs sham and VNS + burn animals). There was no significant difference noted between sham and VNS + burn animals.

Lung ICAM-1 expression levels are normal in VNS-treated animals

ICAM-1 expression on the surface of pulmonary endothelium (Fig 3, *A–D*) was markedly increased in burned animals (*B, arrowhead*) when compared to sham animals (*A*). In contrast, VNS + burn prevented the increase in ICAM-1 expression to levels similar to those of sham animals (*C*). Abdominal vagotomy prior to VNS and burn (*D*) showed ICAM-1 expression similar to burn animals, indicating loss of protective effect and inflammatory signal produced by the pulmonary endothelium.

VNS reduces lung polymorphonuclear leukocyte infiltration

Immunohistochemistry staining for the myeloperoxidase enzyme provides visual evidence of polymorphonuclear leukocyte infiltration (Fig 3, *E–H*). Sham animals (*E*) demonstrate few neutrophils present compared to burn (*F*, *arrows*). VNS reduces the inflammatory signal necessary for PMN infiltration following burn. Consequently, few neutrophils are seen in images taken from VNS-burn animals (*G*). Abdominal vagotomy prior to VNS and burn eliminates the protection offered by VNS and more neutrophils are seen in the lungs similar to burn animals (*H*, *arrows*).

Neutrophil infiltration into the lung tissue can be quantified by counting positively stained cells (Fig 4). Neutrophil counts are low in sham animals compared to burn animals (6.13 ± 1.1 vs 44.9 ± 7 positive cells/10 fields; $P < .001$). VNS + burn significantly reduced the number of positively staining cells within the lungs to numbers similar to sham (5.7 ± 0.6), but cell counts were significantly increased in burn and vagotomy prior to VNS and burn groups compared to the VNS-treated group ($P < .001$).

Myeloperoxidase activity is less in VNS-treated animals

MPO enzymatic activity 24 hours in lung tissue (Fig 5) of sham animals (1.62 ± 0.15 units/mg tissue) was significantly less than in burned animals (3.72 ± 0.58 ; $P < .005$). In contrast, VNS + burn significantly reduced myeloperoxidase activity to levels similar to sham (1.63 ± 0.2) compared to burn and abdominal vagotomy prior to VNS with burn (4.89 ± 0.52 ; $P < .005$).

VNS reduces lung keratinocyte-derived chemo-attractant

Mouse KC (Fig 6) is the analog to human IL-8 responsible for signaling activated neutrophils to infiltrate into inflamed tissues. Sham, uninjured animals produce low levels of KC ($21.49 \text{ pg/mg} \pm 13.1$) compared to burn animals ($657.97 \text{ pg/mg} \pm 77.9$) 6 hours after the thermal insult (*A*, $P < .001$). VNS + burn animals had elevated levels of KC (256.94 ± 66.2) but were not significantly elevated compared to sham. Burn and animals that were subjected to abdominal vagotomy prior to VNS and burn had levels significantly higher than VNS-burn animals ($718 \pm 131.4 \text{ pg/mg}$ vs 256.94 ± 66.2 , respectively; $P < .03$).

Mouse KC was also measured at 24 hours after injury to assess whether VNS may be simply delaying the inflammation as seen in burned animals. Although the trends in comparison of groups were the same as observed at 6 hours, there was no significant difference in KC levels at 24 hours between groups. The increased variability seen suggests that the proportion of inflammatory signal is not changing and that the protective effects of VNS are persistent.

VNS blunts NF- κ B activation

In order to determine the magnitude change of inflammatory signaling, the NF κ B pathway was analyzed (Fig 7). In the inactive state, NF- κ Bp65 is bound to its inhibitor molecule, I κ B- α and resides in the cytoplasm. Accordingly, the level of phosphorylation of I κ B α in the cytoplasm of sham animals is low (*A*). Burn insult led to a significant increase in phosphorylation of I κ B- α within the cytoplasm compared to sham and VNS + burn ($P < .$

0001). When gut protection is eliminated via abdominal vagotomy prior to VNS and burn, levels of P-I κ B- α rise significantly compared to sham and VNS + burn ($P < .0001$). Representative P-I κ B- α Western Blots with β -actin loading controls are shown (A).

Likewise, as a result of its dissociation from I κ B- α , a significant increase in phosphorylated NF- κ B p65 was observed in the lung nuclear extracts of burn animals compared to sham and VNS + burn ($P < .01$; Fig 7, B). Vagotomy prior to VNS and burn removes the protective effect and NF- κ B p65 levels rise similar to burn. Representative blots for NF- κ B p65 from nuclear extract with Lamin B loading controls are pictured in Fig 7, B, as well. In both experiments, no difference in NF- κ B activation was observed between sham and VNS treated animals.

Bioluminescence of NF- κ B

Activation of the NF- κ B pathway in whole lung was also analyzed using transgenic animals (Fig 8). Sham animals demonstrated a very low level of bioluminescence ($1.3 \times 10^5 \pm 31000$ photons/cm²/sr) and appear minimally purple/blue. The bioluminescence of transgenic NF- κ B-luc mice was increased in burn animals ($6.71 \times 10^5 \pm 96000$ photons/cm²/sr), and was significantly elevated compared to sham and VNS + burn ($1.70 \times 10^5 \pm 56000$ photons/cm²/sr; $P = .03$). When abdominal vagotomy is performed prior to VNS and burn, the protective effect of VNS is lost and the bioluminescence of vagotomized animals is significantly increased compared to sham and VNS + burn animals ($6.29 \times 10^5 \pm 20000$ photons/cm²/sr; $P > .03$).

DISCUSSION

The present study is not the first to suggest that vagus nerve stimulation may have an important role in the evolution, and possibly the prevention, of burn-associated, neutrophil-dependent ALI.³⁴ However, this study does specifically outline the mechanisms involved in the development of ALI and utilizes a powerful bioluminescent technology to characterize the involvement of the NF- κ B pathway activation in the signaling process. Song et al³⁴ suggest that severe burn leads to ALI and that vagus nerve stimulation prevents injury by signaling through the $\alpha 7$ nACh receptor. In their study, histology was analyzed only 5 hours after injury, which simply does not allow sufficient time for actual lung injury to occur. Furthermore, the lack of histologic evaluation by a pathologist blinded to the study groups raise the possibility that their findings were more likely due to sample bias and perhaps atelectasis than actual injury due to burn. The authors go on to state that there is significant neutrophil infiltration into the lungs, but provide no objective measures of influx. We have gone further and demonstrated that VNS attenuates the activation of the NF- κ B pathway, decreases pulmonary neutrophil infiltration, and ultimately prevents the histologic changes secondary to burn injury. This is consistent with work previously performed in our laboratory showing that ALI is a neutrophil-mediated process appearing as early as 24 hours after injury in response to activation of the NF- κ B pathway.^{11,12}

Several important conclusions can be drawn from our experiments. While employing vagal nerve stimulation does have a protective effect preventing acute lung injury, this protective effect does not appear to be direct. The surgical abdominal vagotomy does not interrupt any

of the vagus nerve fibers that enter into the pulmonary parenchyma. Thus, one can conclude that if vagal nerve stimulation had a direct effect on the lungs to prevent ALI following burn, then there should be no lung injury if fibers to the lung are intact. Our study clearly demonstrates the contrary. Therefore, ALI is a secondary event that can be prevented as long as the abdominal vagal fibers are intact acting on intra-abdominal organs. Fig 9 visually represents a schematic to illustrate the importance of the intact neuro-enteric axis in prevention of secondary acute lung injury via the gut-lung axis following intestinal barrier breakdown after burn. In addition, VNS as a preventive strategy does not appear to simply delay inflammation as evidenced by the KC levels at 6 and 24 hours in the VNS + burn group.

Recently, evidence has focused on the spleen as the gatekeeper to systemic inflammation, which can be prevented via the “cholinergic anti-inflammatory pathway.”²³ Huston et al²³ suggest that if the spleen is removed, vagus nerve stimulation is unable to further reduce systemic inflammation. Therefore, an intact spleen is required for vagus nerve stimulation to prevent inflammation. We have recently shown that splenectomized animals have the same degree of intestinal injury after burn as normal animals.²⁹ Additionally, we demonstrated that vagus nerve stimulation performed in animals that underwent splenectomy continues to protect the intestinal mucosal barrier from breakdown via activation of enteric glia cells. Combining the ability to prevent intestinal barrier breakdown and the knowledge that activation of intestinal lymph after burn causes acute lung injury,²⁸ we uncover the gut-lung axis and suggest a means to prevent ALI when the intestinal mucosal barrier would otherwise become compromised. It is possible that the anti-inflammatory mechanism occurring locally within the gut is mediated through the $\alpha 7$ nACh receptor, but to date there is not convincing evidence to suggest the presence of that receptor on enteric glia cells. Presently, experiments are underway in our laboratory to answer this specific question.

Traditionally, electron mobility shift assay (EMSA) and Western blot have been used to quantify DNA binding of phosphorylated NF- κ B p65, or presence of phosphorylated NF- κ B p65 within the nucleus, initiating transcription of proinflammatory proteins. These assays are time consuming and prone to operator error. Bioluminescence measurements using NF- κ B-luc transgenic mice allows us to perform the same analysis.³⁵ Since these animals are subjected to the same insults as the nontransgenics, and the only other intervention is an intraperitoneal injection of the luciferin substrate, this technique is quicker, simpler, and significantly less prone to technical error compared to both EMSA and immunoblotting. Bioluminescent imaging has several advantages over traditional imaging techniques and our laboratory is interested in evaluating how this technology can be applied to the study of injury and inflammation. The linear enzyme kinetics of luciferase and peak intensity of light emitted combined with high sensitivity and minimal background allows for real-time imaging,³⁵ making bioluminescence an attractive modality. Bioluminescence is, however, in its infancy and is not without its limitations. For example, because of chest wall quenching of signal, this technique could not be performed in vivo in the current model of ALI imaging. However, with circulating blood providing substrate to the luciferase enzyme up to the moment of sacrifice, the measurements are close to real-time. As the detection

technology improves and the reporter models become more sophisticated, a noninvasive in vivo model should become possible.

In conclusion, we have demonstrated that VNS exerts significant protection against end organ injury following severe burn injury. VNS itself does not appear to have negative hemodynamic effects.^{29,34} In our burn model, there appears to be no direct protective effect with VNS on the lungs. Instead, based on this research and previous work by our laboratory, the importance of the gut-lung axis is revealed. VNS protects the integrity of the intestinal mucosal barrier that, if lost, leads to secondary acute lung injury. If applicable to humans, this could lead to significant possibilities for new therapeutic approaches in the care of the injured patient.

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Fig. 1.

Lung histology at 24 hours. VNS attenuates burn-induced ALI. Lung sections were harvested from animals after 30% TBSA burn ($n = 3$ animals per group) then stained with hematoxylin and eosin. *Black box* denotes the area of the image to the right (*E–H*). Panels *A* and *E* show sections of lung from sham animal at low and high magnification (20 $\times$ and 60 $\times$, respectively). Panels *B* and *F* show lung of burned animals showing increased intra-alveolar hemorrhage (*solid arrow*), thickening of the alveolar membranes (*outline arrow*) and hyaline membrane formation (*arrowhead*). Lung sections taken from animals that underwent right cervical VNS prior to injury demonstrate minimal change compared to sham animals as seen in Panels *C* and *G*. Panels *D* and *H* show sections from animals subjected to abdominal vagotomy prior to VNS and burn. These images demonstrate ALI after the loss of protection from VNS on the gut, indicating that when the gut barrier breaks down, ALI ensues. *Black bar*: 20 μ m.

Fig. 2.

Lung injury score. Lung injury scores were significantly higher in both burn animals and animals subjected to vagotomy prior to VNS and burn. $*P < .05$ vs sham; $tP < .05$ vs VNS + burn ($n = 3$ animals per group).

Fig. 3.

ICAM-1 and MPO immunostaining at 24 hours after burn. ICAM-1 expression in lung sections were low in sham and reduced in VNS + burn animals (Panels A, C), and higher following burn (Panel B) and vagotomy + VNS + burn (Panel D). The arrowhead on Panels B and D show increased ICAM-1 deposition on pulmonary endothelium. Neutrophils were identified after staining for MPO and using DAB substrate kit to produce positively stained cells seen with granular appearance (Panels E–H, solid arrows). All images are for ICAM-1 are at 60 $\times$ magnification and images for MPO are at 40 $\times$. Black bar: 20 μ m.

Fig. 4.

Neutrophil infiltration in lung tissue at 24 hours. Sham animals had very few neutrophils present in the lungs (6.13 ± 1.1 positively stained cells/10 hpf). Neutrophil infiltration was significantly increased in burn animals (44.9 ± 7 positively stained cells/10 hpf) compared to both sham and VNS + burn animals (5.7 ± 0.6 positively stained cells/10 hpf; $*P < .001$). Abdominal vagotomy performed prior to VNS + burn causes a significant increase in neutrophil influx similar to burn (46.2 ± 3.2 positively stained cells/10 hpf; $tP < .001$ vs sham and VNS + burn). $n = 5$ animals per group.

Fig. 5.

MPO activity in lung tissue 24 hours after burn. Myeloperoxidase activity was measured 24 hours after burn and was reduced with VNS prior to burn injury. Sham and VNS + burn groups were similar and were significantly lower than burn alone and when abdominal vagotomy was performed prior to VNS + burn. $*P < .005$ vs sham; $tP > .005$ vs VNS + burn; $n = 5$ animals per group.

Fig. 6.

Keratinocyte-derived chemoattractant (KC) levels in lung tissue. KC was measured by ELISA ($n = 4$ animals per group) 6 and 24 hours after thermal insult. At 6 hours (A) both burn animals and animals subjected to abdominal vagotomy prior to VNS + burn had KC levels significantly higher than sham ($*P < .001$). VNS + burn animals had levels similar to sham. KC levels were significantly lower in VNS + burn animals than in burn and vagotomized animals ($tP < .03$; $tP < .01$). At 24 hours (B), there was no statistical difference between all groups. Although the trends appear the same, there are modest reductions in KC levels 24 hours after injury. $n = 5$ animals per group.

Fig. 7.

Cytoplasmic IκB-α and Nuclear NF-κB p65 phosphorylation in lung tissue. Panels A & B show representative Western blots for cytoplasmic P-IκB-α (A) and nuclear NF-κB p65 (B). β-actin and Lamin B loading controls are also shown to demonstrate relatively equal protein load across all lanes. VNS resulted in a decrease in phosphorylation of IκB-α compared to burn alone and animals subjected to vagotomy prior to VNS + burn at 6 hours (* $P < .001$, Panel C). Burn and vagotomized animals also resulted in greater phosphorylation of NF-κB p65 in burn animals compared to sham and VNS + Burn. (* $P < .001$; $t_P < .01$, respectively; Panel D). $n = 5$ animals per group.

Fig. 8. Bioluminescence from NF- κ B-luc transgenic mice in lung tissue. NF- κ B-luc transgenic mice were used to perform luminescent quantification of NF- κ B activation in the lungs 6 hours after thermal insult. The images are color matched on the same scale for all animals ($n = 5$ animals per group). *Red* signifies more intensity and *violet* signifies lower intensity. Burn insult produced a near 6-fold increase in luminescence over sham and VNS + burn. Abdominal vagotomy prior to VNS + burn significantly increased luminescent signal compared to sham and VNS + burn. * $P = .02$ vs sham; $t_P < .03$ vs VNS + burn.

Fig. 9.

The importance of the gut-lung axis. This diagram visually represents the importance of the gut-lung axis. When VNS is interrupted by abdominal vagotomy, subsequent ALI occurs as a result of disruption of the neuro-enteric axis leading to intestinal barrier breakdown. If the vagus nerve and the neuro-enteric axis are intact, however, subsequent ALI does not occur in a severe burn model.