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Vagus nerve stimulation blocks vascular permeability following burn injury in both local and distal sites

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Abstract

Recent studies have shown that vagus nerve stimulation (VNS) can block the burn injury-induced systemic inflammatory response (SIRS). In this study we examined the potential for VNS to modulate vascular permeability (VP) in local sites (i.e. skin) and in secondary sites (i.e. lung) following burn injury. In a 30% total body surface area burn injury model, VP was measured using intravascular fluorescent dextran for quantification of the VP response in skin and lung. A peak in VP of the skin was observed 24 hours post-burn injury, that was blocked by VNS. Moreover, in the lung, VNS led to a reduction in burn-induced VP compared to sham-treated animals subjected to burn injury alone. The protective effects of VNS in this model were independent of the spleen, suggesting that the spleen was not a direct mediator of VNS. These studies identify a role for VNS in the regulation of VP in burns, with the translational potential of attenuating lung complications following burn injury.

INTRODUCTION

Burn-induced edema and changes in vascular permeability (VP) are a hallmark of severe thermal injury of the skin and secondary organs (i.e. lung, gut, and brain). While established resuscitation protocols exist for the fluid management of burn patients, there remains a significant unmet need to identify novel therapeutic approaches to alter early post-burn injury edema formation in tissues, and minimize the complications of early hypovolemia and massive fluid resuscitation[1–5]. In this study we focused on the VP component of burn-induced tissue edema, and the capacity for parasympathetic nervous system (PNS) to suppress inflammatory responses in peripheral tissues following full thickness burn injury to the skin. Although skin is not directly innervated by the vagus nerve, the skin is in fact an important target for non-neural cholinergic anti-inflammatory signaling mediating epidermal innate immune and epithelial barrier function[6], consistent with a central role for the neuroendocrine regulation of immunosuppression[7]. For example, vagal nerve stimulation (VNS) can reduce inflammation through anti-cholinergic signaling[8, 9] that reduces the expression of inflammatory cytokines and suppresses circulating immune cell responses[7, 10]. The protective effect of VNS on systemic inflammation and secondary organ failure indicates that while the vagus nerve can mediate direct effects on the spleen[11], gut[12] and

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other organs, VNS can also have anti-inflammatory effects on distal organs[7–10, 13–16]. Using a model of cutaneous burn, we show that anti-cholinergic signaling mediated by vagal nerve stimulation affects the vascular integrity (i.e. vascular permeability, VP) of blood vessels in the skin (i.e. margins of the primary injury site), as well as mediating a reduction in the VP of the lung (i.e. a secondary site). Suppressing burn-induced VP through anti-cholinergic signaling presents a novel strategy for the management of burn-related edema.

Significant burn injury initiates a complex physiologic response that leads to an increase in microvascular VP and an accumulation of large amounts of fluid in the cutaneous interstitium both locally at the site of burn injury and systemically, distal to the burn[17]. Alterations in VP after burns are thought to be mediated through a number of factors. For example, histamine, bradykinin, leukotrienes, substance P, and nitric oxide can all cause endothelial cell activation, which can lead to protein loss, oncotic/osmotic pressure changes and uncontrolled interstitial tissue edema[8–10]. Increased VP in burn models has been studied and established through modalities such as wet to dry ratios, radio-labeled iodine tracers, and Evan's blue dye (EBD) in efforts to identify possible therapeutic targets to prevent massive tissue edema and prevent the morbidity and mortality that ensues following burn[5, 18–20]. Here, we use EBD as a tracer that binds to serum albumins to validate our animal model, with focus on the use of fluorescent dextran, which has a superior dynamic range and signal-to-noise, that can be imaged to localize changes in VP.

In the studies described here, we determined first whether VNS affects endothelial barrier integrity in skin adjacent to a skin injury site (i.e. burn margin) and reduces burn-induced VP in the area adjacent to the burn wound (i.e. central burn). We demonstrate that VNS blocks burn-induced VP 24 hours post-burn injury (PBI). Burn-induced VP was assessed with a 70 kDa fluorescent dextran tracer to enable non-invasive serial and quantitative imaging of the full thickness burn region as well as a qualitative assessment of skin margins. Second, we examined the translational potential of the VNS-mediated protection of the burn-induced VP response on secondary organs (i.e. lung). We observed that the increase in lung VP following burn injury could be blocked by VNS, thus supporting the translational potential of VNS in mediating lung edema in this SIRS model.

METHODS

Thermal Injury Model

Male balb/c mice weighing 24–28 grams were obtained from Jackson Laboratories (Sacramento, California). Animals underwent dorsal and ventral fur clipping with an electric clipper, were anesthetized with inhaled isoflurane, and subjected to a 30% total body surface area (TBSA) dorsal steam burn for 7 seconds using a template designed to estimate 30% TBSA¹⁶. All animal groups received a subcutaneous injection of normal saline (1 mL) and buprenorphine (100µL of 12.6 µg/mL) in a non-burned area for fluid resuscitation and pain control. 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 followed by right cervical VNS was performed on select animals immediately prior to the thermal insult. Intermittent stimulation of the vagus nerve was performed using a VariStim III probe (Medtronic Xomed, Jacksonville, FL) at 2 mA for 10 minutes. Following nerve stimulation, the incision was closed with a running 4-0 silk suture. Sham animals underwent right cervical incision and exposure of the vagus nerve but did not receive electrical stimulation.

Tissue Harvest

Animals were sacrificed at 6 and 24 hours following injury and tissues were harvested. Four 8mm full thickness skin biopsies were obtained from three sites: 1) the central burn zone, 2) the burn margin adjacent to the central burn, and remote zone (chest and abdomen). Skin biopsies were fixed in 10% buffered formalin (Richard Allan Scientific, Pittsburgh, PA) for histologic evaluation and immunohistochemistry.

Splenectomy

A cohort of animals underwent splenectomy immediately prior to VNS and thermal insult. Prior to the splenectomy, abdomens were shaved and sterilized with 10% betadinen and the spleen removed through a small upper midline incision. The vascular pedicles were identified, ligated with 2-0 silk, and the abdomen was closed in one layer with a running 4-0 silk suture.

Histologic Evaluation

Full thickness 8 mm skin biopsies were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. UCSD Histology Core Services performed Hematoxylin & Eosin staining of skin biopsies. Sections were viewed via light microscopy and the degree of burn injury evaluated by a pathologist.

Vascular Permeability Assay with Evan's Blue Dye (EBD)

To determine the amount of EBD in the experimental area, 150 μ L of a 133 mg/ml EBD solution deionized water was administered through tail vein injection at either 6 hours or 24 hrs following thermal injury. EBD-injected mice were maintained for 30 minutes and subjected to sacrifice. Two 8 mm full-thickness skin biopsies were excised from: 1) the central burn; 2) the burn margin, and 3) the distal zone of each animal group. A similar procedure and time schedule was used for all animals. Each sample was placed in 500 μ L of formamide and incubated for 24 hr in a heat plate at 65°C to extract EBD from the tissue. The amount of tissue EBD was quantified by a spectrophotometer at an absorption of 650nm[18, 21, 22]. Three measurements were performed on each sample and the mean value was used for calculations.

Vascular Permeability Assay with 70 kDa FITC Dextran

Mice were subjected to a tail vein injection with 150 μ L (50 mg/ml in PBS) 70 kDa FITC dextran (FD70) (Sigma, St. Louis, MO) and sacrificed 30 minutes later. Four 8 mm skin biopsies from the central burn, burn margin, and remote skin were obtained. Similarly, lung samples were collected and imaged ex vivo. Skin and lung biopsies were imaged to measure VP to FD70 with a deep cooled CCD imaging system equipped with appropriate fluorescence filter cubes with background subtraction. Images were analyzed using Living Image software version 4.0 (IVIS Spectrum, Caliper Life Sciences, Hopkinton, MA).

Statistical Analysis

A Student t-test was used for analysis of VP data from the burn models with a minimum of 4 samples per group where P values of less than 0.05 being statistically significant.

RESULTS

Characterization of animal model to assess burn-induced VP

To demonstrate the histological characteristics of our full-thickness cutaneous 30% TBSA injury model, we subjected representative skin samples from the burn margin (Figure 1A)

and central burn zone (Figure 1B) to staining with hemotoxylin and eosin. The histology of this full thickness injury was characterized by a markedly decreased healthy vasculature, the presence of small vessel thrombosis, severe damage to sebaceous glands, the loss of hair follicles, the loss of epidermal layer integrity, and collagen hyperplasia with increased deposition in the dermis (Figure 1B).

We used a classic dye (i.e. EBD) to demonstrate a burn-induced change in cutaneous VP. A significant increase was observed in the burn margin compared to the sham and central burn skin (Figure 1C). Furthermore, an increase in skin VP was observed in skin distal to the central burn on the ventral side of the same animal (Figure 2). These results suggested that the post-burn injury (PBI) VP response was systemic and extended beyond the VP identified in the burn margin.

Next, a 70 kDa fluorescent dextran (FD70) with a higher signal-to-noise than EBD was used in the same burn model where FD70 was detectable using a digital imager, quantifiable in fresh *ex vivo* tissues, and imaged to assess local vs. regional changes in FD70 dye with respect to the injury site (Figure 2). Based on these parameters, we examined burn-induced localization of the FD70 to examine distinct zones of VP based on the burn site.

Measurement of FD70 in 8 mm skin biopsies collected from the burn margin vs. the central burn site demonstrated a maximum of 3 fold increase in FD70 24 hours PBI, ($1.99^9 \pm 1.21^8$ in burn margin vs. $5.67^8 \pm 9.87^7$ in sham, Figure 2). The kinetics of PBI-induced VP were assessed by quantification of FD70 in the margins of the burn wound at 6 hours and 24 hours PBI (Figure 2). While significant increases were observed in 6 hours (i.e. between the burn margin and sham), the highest levels were observed at 24 hours where an increase in VP was observed in the burn margin and the distal skin compared to sham. These findings demonstrated that cutaneous burn induced both a local (i.e. burn margin) and systemic increase (i.e. distal skin) in VP.

Vagus Nerve Stimulation attenuates burn-induced skin VP

We used the 30% TBSA skin injury model to determine whether VNS was protective. In this model, mice were subjected to VNS stimulation followed by steam burn injury. Representative *ex vivo* skin samples of the burn margins and remote skin are shown in Figure 3 and demonstrate the focal PBI-induced localization of 70FD in the burn margin and remote skin. Quantification of 24 hour PBI-induced VP revealed a statistically significant 2.6 fold decrease in the VP of the burn margins of burn animals vs. burn animals treated by VNS (Figure 4). Analysis of skin VP in burn-induced animals subjected to VNS demonstrated a statistically significant decrease of VP at the burn margin (Figure 4A), as well as in distal skin (Figure 4B). We next evaluated whether the absence of the spleen could uncouple the VNS-mediated protection observed in the burn model. Mice were subjected to splenectomy, followed by VNS and burn injury. The VP of the burn margin (Figure 4A) and remote skin was then determined (Figure 4B). We did not observe a significant difference between the burn/VNS and the burn/VNS/splenectomy groups, suggesting that the VNS-mediated protection was spleen-independent. Using this VP model, these data show that VNS mediated not only reduced VP at the burn margins immediately adjacent to the injury site, but also reduced VP in remote skin suggesting that VNS has a systemic effect on mitigating the inflammatory response affecting VP that ensues after burn injury.

VNS blocks burn-induced VP of lung

To determine whether the protective effects of VNS on distal skin extended beyond the dermis to secondary sites, we examined burn-induced VP in lung. At 24 hr PBI, we observed a 4.8 fold increase in VP of lungs vs. sham controls (Figure 5) that was blocked by

VNS prior to burn injury. Prior splenectomy did not affect VNS-mediated lung protection, consistent with our observations of spleen-independent VNS-mediated reduction of burn-induced VP of the skin. These studies demonstrate that the translational potential of VNS-mediated effects on VP extend beyond the skin and have the potential to affect secondary organs.

DISCUSSION

The global tissue edema and increased VP that follows burn injury continues to cause significant morbidity and mortality in burn patients, leading to complications related to early hypovolemia and massive fluid resuscitation[1–3]. Our study indicates that the consequences of cutaneous injury in experimental models can be blocked by the systemic anti-inflammatory properties of VNS. The spleen-independent effects of VNS in the burn model was similar to our previous studies in burn models where the burn-induced gut barrier breakdown was spleen independent through direct vagal nerve innervation[13], but in contrast with the spleen-dependent VNS protection observed in models of endotoxemia[8–10].

In the late 1990's, a cholinergic anti-inflammatory pathway mediated through the vagus nerve was identified as essential in delivering information that regulates the inflammatory response mounted by a host in response to infection or injury. Direct electrical stimulation of the vagus nerve significantly inhibits cytokine production by innate immune cells in the spleen, liver, gastrointestinal tract, heart and other tissues that are innervated by the vagus nerve[7]. Secondary organ injury, such as acute lung injury, is also attenuated indirectly via VNS and abolished in vagotomized animals[23]. Previous studies have examined the capacity for afferent vagal nerves and C fibers to mediate the kinetics of the wound healing response and link systemic stress with cutaneous disease[6, 24]. Based on evidence linking innervation with the revascularization of sites of injury, we considered whether VNS, although not directly innervating the dermis, may mediate vascular responses indirectly through hemostatic mechanisms or regulation of innate immune cell response[16, 25].

In vitro and *in vivo* animal models demonstrate that cholinergic stimulation blocks endothelial cell activation and has an important role in regulating leukocyte recruitment during inflammation and infection although these effects were observed using nicotine levels approaching LD50[15]. In contrast, VNS mediates anti-inflammatory signals through an innate immunity reflex[7] without adversely affecting blood pressure variability[13]. In this study we have shown that activation of the parasympathetic nervous system reduced VP of skin and lung suggesting that the systemic effects of VNS can block the local and systemic sequelae of increased VP. Regulation of cutaneous injury through the central nervous system suggests that there may be additional pathophysiological relevance of managing burn-induced VP through VNS. Here we have determined the kinetics of vagus nerve-mediated protection and demonstrated proof of principle in burn-induced VP.

The recognition of VP as an important sequelae of burn injury dates back over 60 years when Drinker, McMaster and colleagues demonstrated the existence of burn-dependent changes in the cutaneous vascular bed[26–28]. The changes in capillary VP after thermal injury have been studied using models that have focused on the composition and flow of lymph[1, 27, 29], vascular integrity through dyes and dextran molecules[30, 31], through radioactively tagged albumin[4], and wet to dry weight measurements on tissue biopsies[32–34]. We have previously shown the utility of invasive and non-invasive imaging fluorescent dextrans in animal models of full thickness wound/grafting models to define the kinetics of VP[35] and vascular remodeling with a focus on the angiogenic rim around the injury site[36]. In this study we use a similar technical approach to now determine whether the

vascular remodeling in the burn injury site can be affected by vagus nerve input. This study is the first to identify the effects of VNS on cutaneous VP changes in a burn model.

The anti-inflammatory pathway controlled by the vagus nerve is clearly an important mechanism modulating the host response to injury in multiple injury models including endotoxemia, peritonitis, pancreatitis, hemorrhagic shock, arthritis, myocardial infarction and colitis[7, 8, 10, 13, 14, 16, 23–25, 37]. The local and systemic effects of modulating the innate immune responses by VNS have also been studied to elucidate the mechanisms that mediate this anti-inflammatory response. A strong body of literature supports the hypothesis that the systemic production of pro-inflammatory cytokines is suppressed through the activation of α -7 nAChR[8, 38]. This receptor is expressed in human microvascular endothelial cells[39], but the exact link between vagus nerve and the local and systemic inflammatory response remains unclear.

Previous studies have shown that burn injury can induce edema of intact skin, muscle, and internal organs that are distant to the locally injured skin. This suggests that a generalized increase in capillary permeability is mediated by circulating factors[33]. Burn-induced intestinal and lung injury are also a supplementary source of inflammatory cytokines that may contribute to cutaneous burn-induced VP response.

It is of particular interest to note that the spleen has been previously identified to influence cutaneous wound healing[40, 41]. While it is clear that the spleen has an important role in immuno-modulation responses to injury[11], our studies in a burn model indicate that the spleen is not required for the vagus nerve-mediated protection of distal organ injury in the gut and lung following burn injury[13]. The ability of the VNS to significantly attenuate systemic burn-induced VP might be predictive of multiple organ failure. If so, the anti-inflammatory effect of VNS could potentially change therapeutic paradigms in burn resuscitation through the development of vagal nerve agonists.

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Figure 1C

Figure 1. Histology of the cutaneous burn model

A) Histology of normal mouse balb/c skin with a hematoxylin/eosin stain showing an intact epidermal cell layer (1), hair follicles and sebaceous glands (2) and normal keratinocytes and dermal collagen (3). **B)** Histology of full-thickness steam burn of mouse skin showing interrupted epidermal cell lining (1), damaged hair follicles (2), thickened and scarred dermal collagen layer (3), and thrombosed capillaries (4). **C)** Quantification of burn injury-induced VP using Evan's Blue Dye (EBD) extracted from skin 24 hours post-burn injury. Comparison of EBD VP revealed a significant increase in the VP of the burn margin compared to sham skin or the central burn (*, SEM, $p < 0.05$, $n=4$).

Figure 2. Burn-induced VP using fluorescent dextran

Quantification of burn injury-induced VP using fluorescent 70 kDa FITC dextran (70FD). Comparison of VP between the burn margin with sham skin revealed a significant increase at 6 and 24 hours PBI compared to sham skin, with a peak at 24 hours PBI (*, SEM, $p < 0.05$, $n=4$). There was no significant difference in burn-induced VP between central burn and sham skin.

Figure 3. Vagal nerve stimulation (VNS) reduces burn-induced cutaneous VP

Representative *ex vivo* images of the 8 mm skin punches flanking the burn margin (upper row) and distal skin (bottom row) were used for quantification with 70FD. Samples shown are from sham animals (A and E), animals subjected to VNS followed by burns (B and F), animals subjected to burn (C and G), and animals subjected to a splenectomy followed by VNS and burn injury (D and H). Samples were isolated and imaged with an IVIS Spectrum as described in the Materials and Methods. Quantification of data sets is presented in Figure 4.

Figure 4. Quantification of VNS-mediated reduction in burn-induced VP of the skin

A) The burn margins of sham, burn+VNS, burn, and splenectomized burn+VNS mice were assayed for FD70 VP 24 hours PBI. A significant reduction in VP was observed in the margins of the burn+VNS group compared to the burn group (*, SEM, $p < 0.05$, $n=6$). There was no significant difference in VP in the group subjected to VNS prior to burn injury when compared to sham demonstrating VNS-mediated reduction in VP. The group subjected to splenectomy followed by burn+VNS had similar reduction in VP to the burn+VNS group (*, SEM, $p < 0.05$, $n=6$) suggesting that the effects of VNS were spleen-independent. **B)** Similar to the studies of burn-induced VP quantified in the margin in (A), a reduction in the VP of distal skin was observed in the burn+VNS group when compared to the burn group ($p =$

0.079). VNS reduced burn-induced VP to the levels of sham skin. Splenectomy of burn +VNS animals did not affect the VNS-mediated reduction in VP.

Figure 5. Quantification of VNS-mediated reduction in burn-induced VP of the lung

Mice were subjected to splenectomy (Splx), VNS, or burn injury as indicated, and assayed for lung VP as described in the Materials and Methods. A significant 4.8 fold increase in lung VP was observed 24 hours PBI, that was blocked by VNS administered prior to burn injury (*, SEM, $p < 0.05$, $n = 6$). Splenectomy of burn+VNS animals did not affect the VNS-mediated reduction in VP.