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CPSI-121 pharmacologically prevents intestinal barrier dysfunction after cutaneous burn through a vagus nerve-dependent mechanism

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Abstract

BACKGROUND—We have recently demonstrated the protective effects of electrical stimulation of the vagus nerve in prevention of gut injury after severe burn. Here we evaluate the potential for a pharmacologic agonist of the vagus nerve as an approach to regulate outcomes in preclinical models. We tested a new generation of guanyldiazide-derived compounds, CPSI-121; a compound that may activate the parasympathetic nervous system through poorly understood mechanisms to determine whether we could prevent intestinal mucosal barrier breakdown.

METHODS—Male balb/c mice were subjected to a full-thickness, 30% total body surface area steam burn, and the efficacy of CPSI-121 was tested against vagus nerve stimulation (VNS) postburn at 4 hours. Surgical vagotomy was used to disrupt the neuroenteric axis and gut injury prevention was assessed. Gut barrier dysfunction was quantified by permeability to 4-kDa fluorescein isothiocyanatedextran. Gut injury was assessed by histologic evaluation. Tight junction protein expression (ZO-1 and occludin) was characterized by immunofluorescence and immunoblot.

RESULTS—VNS and CPSI-121 administration significantly reduced the permeability to 4-kDa fluorescein isothiocyanate-dextran and maintained normal histology compared with burn.

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AUTHORSHIP

M.K. was responsible for data collection, data synthesis, and primary manuscript preparation. Y.O.-P., N.L., and L.G.R. assisted in animal experiments and data generation. G.C. aided in the organization of the data and performed immunostaining. P.W. reviewed the histology and generated the raw data for the gut injury scores. B.E., A.B., and V.B. assisted in organization of the data and synthesis of the manuscript. R.C., as the senior author, provided direction for the entire project, reviewed the data, and assisted in writing and reviewing the manuscript.

DISCLOSURE

The authors declare no conflicts of interest.

However, abdominal vagotomy eliminated the protective effects of both VNS and CPSI-121. ZO-1 and occludin expression was similar to sham in VNS and CPSI-121-treated burn animals, but significantly altered in burn-vagotomized animals. Splenectomy did not alter the effect of CPSI-121.

CONCLUSION—Similar to direct electrical VNS, CPSI-121 effectively protects the intestinal mucosal barrier from breakdown after severe burn. We suggest that this could represent a noninvasive therapy to prevent end-organ dysfunction after trauma that would be administered during resuscitation.

Keywords

Guanyldiazide; gut injury; burn; tight junction; intestinal barrier; trauma

The importance of the intestinal mucosal barrier in protecting the host against resident commensal organisms is paramount to homeostasis and health. Several investigators have hypothesized that the intestine serves as an inflammatory organ after trauma, sepsis, and hemorrhagic shock and intestinal barrier dysfunction leads to multiple organ failure and death.^{1–3} The benefits of electrical stimulation of the vagus nerve to activate the anti-inflammatory effects of the parasympathetic nervous system were first described by Borovikova et al.⁴ Further study into the mechanism of signal transduction pointed to suppression of inflammatory mediator release by splenic macrophages after stimulation of the alpha-7 nicotinic acetylcholine receptor.^{5–7} We have demonstrated an expanded role for vagus nerve stimulation (VNS) and the parasympathetic anti-inflammatory mechanism that provides a local protective effect on the gut, independent of the spleen, that is mediated through enteric glia cells and their secreted product, S-nitrosoglutathione.⁸

Anti-inflammatory strategies using VNS that were applied to the gut have successfully prevented secondary inflammation and injury to other organ systems.⁹ In this schema, when the neuroenteric axis is activated by VNS in a burn model, secondary acute lung injury is prevented. However, if abdominal vagotomy is performed, protective effects of VNS are eliminated and secondary acute lung injury occurs via the gut-lung axis.⁹

We have previously demonstrated that stimulation of the vagus nerve prevents the breakdown of the intestinal mucosal barrier when performed both before and after severe cutaneous burn.^{10,11} The ultimate target of protection is tight junction protein expression in epithelial cells and preservation of their function in the intestinal mucosal barrier.

Evidence supporting the use of vagal nerve stimulation as an anti-inflammatory strategy in burn and sepsis is compelling in small animal models. The bulk of the literature, however, uses focused direct electrical stimulation of the vagus nerve itself, which does not translate well to clinical application in acutely injured patients. A pharmacologic candidate who results in the same effective protection without exposure of the vagus nerve would be more applicable and feasible. Semapimod (or CNI-1493), for example, has shown efficacy as a vagomimetic that can suppress inflammatory cytokines and systemic inflammation by directly activating the vagus nerve.^{12–14}

In this series of experiments, we used CPSI-121 in place of VNS to determine its effect on the intestinal epithelial barrier function in our burn model. CPSI-121 is a guanylhyazone analog, which shares very similar structure and function to CNI-1493. We hypothesized that CPSI-121 would prevent intestinal barrier breakdown similar to VNS and require an intact neuroenteric axis to provide such effect.

MATERIALS AND METHODS

Materials

CPSI-121 is a divalent guanylhyazone that was generously donated by Cytokine Pharmasciences (King of Prussia, PA). Four kilodalton fluorescein isothiocyanate (FITC)-dextran was obtained from Sigma-Aldrich (St Louis, MO). Anti-occludin and ZO-1 antibodies were obtained from Zymed (Carlsbad, CA). Secondary antibodies goat anti-rabbit Alexa-Fluor 488 and horseradish peroxidase-linked anti-rabbit immunoglobulin G was obtained from Invitrogen (Carlsbad, CA) and Cell Signaling (Danvers, MA).

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 a 30% total body surface area (TBSA) 7 second dorsal steam burn.¹⁵ After burn, animals received a 1.4-mL subcutaneous injection of normal saline containing 0.1 mL buprenorphine in a nonburned area for fluid resuscitation and pain control. At that time, cohorts of animals received an intravenous (IV) injection of CPSI-121 dosed at 1 mg/kg diluted in 150 μ L of sterile water. All animals not receiving CPSI-121 were subjected to IV injection of 150 μ L of sterile water. An additional group of animals underwent splenectomy before burn with subsequent drug treatment. For these animals, the abdomen was shaved and sterilized with iodine solution. Through a small upper midline incision, the spleen was eviscerated, the vascular pedicles were identified and suture ligated with 2-0 silk, and the abdomen was closed in two layers with running 4-0 silk suture. 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 to expose the right cervical vagus nerve so as not to cause extensive injury by manipulation for ligation. This was followed by right cervical VNS, performed on selected animals immediately after thermal insult. Stimulation of the 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. Control animals underwent right cervical incision with vagus nerve exposure and 1 mg/kg IV injection of CPSI-121 only. All injections were done via tail vein injection.

Tissue Harvest

Animals were killed 4 hours after injury and tissues were 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 embedding media (OCT; Sakura Finetek, Torrance, CA) for histologic evaluation and immunofluorescence staining.

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 using a scoring system that characterized gut injury on a scale from 0 to 4 with 0 = normal, no damage; 1 = mild, focal epithelial edema; 2 = moderate, diffuse swelling and necrosis of the villi; 3 = severe, diffuse pathology of the villi with evidence of neutrophil infiltration in the submucosa; and 4 = major, widespread injury with massive neutrophil infiltration and hemorrhage as previously described.¹⁶

Intestinal Permeability Assay

An in vivo intestinal permeability assay was performed to assess intestinal barrier function. 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 FITC-dextran (25 mg/mL) 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 was closed. Animals were 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 10,000 g 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.

Confocal Microscopy

Segments of distal ileum were embedded in OCT media and stored at -80°C or paraffin. OCT sections of intestine were cut 10 μm 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 and washed with PBS. 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% bovine serum albumin (BSA) in $1\times$ PBS (BSA; Sigma-Aldrich). The sections were then incubated overnight in the occludin or ZO-1 antibody at 4°C . The following day, they were treated with Alexa Fluor 488 secondary goat

anti-rabbit antibody in 1% BSA for 1 hour at room temperature. Prolong Fade (Invitrogen) was added on 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.

Occludin Expression

Segments of distal small intestine harvested from animals at 4 hours after burn 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 were collected, and the protein concentration of each sample was measured using the bicinchoninic protein assay (Pierce). 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% Tris-glycine gradient gels (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked with 5% BSA in Tris-buffered saline/Tween 20 for 1 hour. Membranes were then incubated overnight at 4°C in primary anti-occludin antibody 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 immunoglobulin G secondary antibody before application of the Supersignal West Pico Chemiluminescent Kit (Pierce Biotechnology) 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.

Statistical Analysis

Data are expressed as the mean $\pm$ the standard error of the mean. The statistical significance among groups was determined using analysis of variance with Bonferroni correction where appropriate. Statistical significance for gut injury was performed using the Kruskal-Wallis test for non-parametric data with post hoc Wilcoxon-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

CPSI-121 Prevents Intestinal Mucosal Injury

Sections of distal ileum from sham and control uninjured animals were compared with those that were subjected to 30% TBSA burn with, and without, administration of CPSI-121, vagal nerve stimulation, splenectomy, and abdominal vagotomy. Histologic sections from sham animals receiving only IV sterile water were similar to animals receiving IV CPSI-121 at the highest dose tested (Fig. 1, A and B). This demonstrated absence of deleterious effects of CPSI-121 alone on the gut epithelium. In contrast, Figure 1, C demonstrates the histologic pattern observed after burn injury with villous tip necrosis, blunting, and sloughing of villi. When CPSI-121 injection or VNS was performed immediately after the burn insult,

there was minimal if any evidence of histologic injury (Fig. 1, D and E). The same normal pattern of hematoxylin and eosin staining was observed in animals subjected to splenectomy before the burn injury followed by CPSI-121 administration. This demonstrated that CPSI-121 provided a protective effect independently of splenic innervation (Fig. 1, F). In contrast, when abdominal vagotomy was performed and as such the neuroenteric axis was interrupted, both VNS and CPSI-121 failed to prevent the histologic changes induced by burn injury in the gut (Fig. 1, G, and H). Taken together, these data demonstrated that an intact vagus nerve was necessary for the biological effect of CPSI-121.

Gut injury scores were significantly increased in burn animals without injury protection. Sham and control animals had similar injury scores with both groups having an average score of 0 of 4. Animals subjected to burn alone had an average injury score that was significantly higher than both sham and control groups (1.78 ± 0.19 vs. 0; $p < 0.001$). Burned animals had significantly higher scores than animals that were treated with VNS, CPSI-121, or had splenectomy (1.78 ± 0.19 vs. 0, 0.4 ± 0.7 , 0; $p < 0.01$). Surgical abdominal vagotomy in the presence of VNS and CPSI-121 eliminated the protective effects with results similar to burn alone (2 ± 0.33 , 2 ± 0.33 vs. 1.78 ± 0.19).

Intestinal Permeability Is Reduced With CPSI-121 Administration

CPSI-121 in the absence of injury did not cause an increase in intestinal permeability (sham $12.42 \text{ ng/mL} \pm 3.92 \text{ ng/mL}$ vs. control $12.68 \text{ ng/mL} \pm 4.73 \text{ ng/mL}$; $p = 1$; Fig. 2). Animals in the burn group had an increase in permeability when compared with sham ($194.22 \text{ ng/mL} \pm 19.47 \text{ ng/mL}$ vs. 12.42 ng/mL ; $p < 0.002$). CPSI-121 and vagal nerve stimulation both protected against increased intestinal permeability after the burn insult ($16.68 \text{ ng/mL} \pm 3.78 \text{ ng/mL}$ vs. $19.86 \text{ ng/mL} \pm 4.82 \text{ ng/mL}$). Splenectomy performed before the burn insult did not alter the protective effect of CPSI-121, which was still observed when compared with burn alone ($32.56 \text{ ng/mL} \pm 11.1 \text{ ng/mL}$ vs. $194.22 \text{ ng/mL} \pm 19.47 \text{ ng/mL}$; $p < 0.007$), indicating that the protective effect of CPSI-121 was spleen independent. However, when abdominal vagotomy was performed before burn, the intestinal permeability of animals undergoing either vagal nerve stimulation or CPSI-121 was indistinguishable from burn alone ($164.38 \text{ ng/mL} \pm 45.56 \text{ ng/mL}$ and $173.45 \text{ ng/mL} \pm 43.98 \text{ ng/mL}$ vs. $194.22 \text{ ng/mL} \pm 19.47 \text{ ng/mL}$). These data indicated that vagal nerve stimulation and CPSI-121 can only offer protection to the gut in the presence of intact neuroenteric innervation.

CPSI-121 Prevents Widening of Intercellular Tight Junctions

We used immunofluorescent staining of the tight junction protein, ZO-1, to assess the integrity of intestinal tight junctions. As seen in Figure 3, A, the gut of sham animals demonstrated close proximity of the cell membranes with no discernable intercellular space. Animals receiving CPSI-121 alone also demonstrated similar staining pattern, indicating that CPSI-121 alone did not disrupt cell-cell integrity (Fig. 3, B). In burn animals, intercellular gaps appear between the intestinal epithelial cells 4 hours after injury (Fig. 3, C, *arrows*). Animals treated with CPSI-121 or vagal nerve stimulation immediately after burn (Fig. 3, D and E) showed preservation of the cytoskeleton and cell-cell integrity. Indeed, the protection offered by CPSI-121 after burn injury and the maintenance of the intestinal epithelial tight junctions were also observed in the absence of the spleen (Fig. 3, F). In contrast, animals

undergoing an abdominal vagotomy before the burn insult, no protection was afforded by vagal nerve stimulation or CPSI-121 to the intestinal mucosa, evidenced by the presence of readily apparent gaps between villous epithelial cells (Fig. 3, G and H, *arrows*).

The paracellular spaces that were clearly visible in burn animals and in those undergoing an abdominal vagotomy were quantified by confocal microscopy (Fig. 4). Significantly larger spaces between cells were observed in animals that were burned and did not receive vagal stimulation or CPSI-121 or underwent abdominal vagotomy when compared with all other groups ($p < 0.05$). CPSI-121 and VNS seemed to equally protect against cell separation. In addition, the observed protection is similarly lost when the continuity of the neuroenteric axis is interrupted by a surgical abdominal vagotomy.

CPSI-121 Prevents Degradation of Intestinal Occludin Expression

A second tight junction protein, occludin, undergoes protein expression alterations in response to severe burn injury.^{8,10,11} Exposure-matched fluorescent intensity correlated to the amount of occludin protein expression after immunostaining (Fig. 5). Sham animals had robust occludin expression (Fig. 5, A) that was unaffected by CPSI-121 alone (Fig. 5, B). After burn, animals showed a reduction in occludin expression evidenced by a lower fluorescent intensity at the cell periphery (Fig. 5, C, *arrow*), which was prevented by IV CPSI-121 or vagal nerve stimulation (Fig. 5, D and E, respectively). In the absence of the spleen, CPSI-121-treated animals had occludin expression similar to their nonsplenectomized counterparts (Fig. 5, F). The disruption of the neuroenteric axis by abdominal vagotomy led to marked reductions in occludin expression when compared with vagal nerve stimulation and CPSI-121-treated animals (Fig. 5, G and H, *arrows*).

These results were confirmed by immunoblotting for the occludin protein in intestinal tissue lysates (Fig. 6). When compared with the average relative band density of sham animals, burn animals had significantly lower occludin expression ($p < 0.05$). In contrast, animals that received CPSI-121 or underwent vagal nerve stimulation maintained a significantly higher level of occludin expression compared with burned animals ($p < 0.05$). When the neuroenteric axis was disrupted with abdominal vagotomy, neither vagal nerve stimulation or CPSI-121 administration successfully maintained occludin protein expression when compared with sham or animals with an intact vagus nerve ($p < 0.05$).

DISCUSSION

Electrical stimulation of the vagus nerve has demonstrated numerous anti-inflammatory and protective effects against organ dysfunction in burn, sepsis, and traumatic brain injury.^{7–11,16} The difficulty in translating these findings to man is that direct electrical stimulation of the vagus nerve is impractical in acutely injured patients. Therefore, a more clinically desirable method would involve a pharmacologic approach. Ideally, a drug that increases vagus nerve output would be administered during the resuscitative phase of trauma care.

In this set of experiments, we administered CPSI-121 during postburn resuscitation, established its efficacy, and compared its effects with direct VNS. We demonstrated that

CPSI-121 at a dose of 1 mg/kg is as effective as VNS in preventing intestinal barrier breakdown after a 30% TBSA burn insult. Furthermore, we have shown, as a proof of concept, that its biological effect is dependent on an intact vagus nerve. CPSI-121 effectively prevented histologic injury of the gut mucosa and maintained intestinal tight junction protein expression and function. Its protective effects were eliminated by disrupting the neuroenteric axis via surgical abdominal vagotomy.

In other models of injury, investigators have argued that the spleen is critical to the function of the cholinergic anti-inflammatory pathway.⁷ Costantini et al.⁸ have shown that gut injury occurs regardless of the presence or absence of the spleen and VNS protects the gut independent of the presence of the spleen. This is because the protection offered to the intestine via stimulation of the vagus nerve seems to be mediated through enteric glia cells and their secreted product S-nitrosoglutathione. In the present experiments, we show that CPSI-121 protects the gut in both the presence and absence of the spleen, presumably by the same mechanism. These findings confirm that the anti-inflammatory effects of VNS are independent of the spleen in the burn model. It is possible that CPSI-121 exerts its protective effects by increasing blood flow of the intestine. Although we have not directly measured any effects on intestinal blood flow after VNS or CPSI-121 administration, we have measured systemic pressure with invasive monitoring in response to VNS and found no effect on systemic blood pressure.⁸

The exact mechanism of action for guanylylhydrazone compounds remains unclear. One guanylylhydrazone, CNI-1493, was clinically shown to be efficacious in reducing systemic tumor necrosis factor- α during high-dose interleukin-2 treatment for renal cell carcinoma and melanoma and reducing the inflammation associated with Crohn's disease.^{17,18} Because of its success in reducing inflammation, we sought to apply a guanylylhydrazone analog to a mouse burn model and determine its efficacy in reducing local gut inflammation and intestinal barrier breakdown. To our knowledge, this is the first time that CPSI-121 has been used in vivo.

In these experiments, CPSI-121 was administered intravenously and successfully maintained low gut injury scores after burn, reduced permeability of the distal ileum to 4-kDa FITC-dextran, and maintained normal expression of the tight junction proteins occludin and ZO-1. We also demonstrated that this biological effect is dependent on an intact vagus nerve. Disrupting the neuroenteric axis via surgical abdominal vagotomy abolished any protective effect of CPSI-121. There is evidence to suggest that this class of compounds increases vagal nerve efferent output in experimental models of inflammation. Borovikova et al.¹³ demonstrated that IV administration of CNI-1493 was effective at increasing vagal impulses for a 10- to 14-minute duration. It is therefore reasonable to infer that CPSI-121 may have a similar effect. Indeed, Bernik et al.¹⁴ demonstrated the anti-inflammatory effects of CNI-1493 in an endotoxemic shock model could be blocked by peripheral surgical vagotomy. This evidence supports our findings that guanylylhydrazone compounds exert their anti-inflammatory effects in a vagus nerve-dependent mechanism.

Although this is the first time CPSI-121 has been used in vivo, this study is not the first to suggest that novel resuscitation strategies can protect against intestinal injury. Deree et al.¹⁹

demonstrated that changing the resuscitative fluid from a standard resuscitative fluid to hypertonic saline and adding pentoxifylline reduced gut inflammatory markers in a preclinical hemorrhagic shock model. There is also strong evidence to support the use of anti-inflammatory medications as adjuncts to trauma resuscitation after burn. Pentoxifylline has demonstrated efficacy in reducing inflammatory pathway activation and cytokine production when administered during resuscitation after thermal insult with concomitant protection of intestinal tight junction proteins.^{20,21} Pharmacologic adjuncts to resuscitation to include pentoxifylline and CPSI-121 given after trauma provide impetus for broadening the field of study in trauma resuscitation. Advances in resuscitative fluids and drug candidates are at the forefront of trauma research and may represent the elusive preventive measure that could eliminate multiple organ failure after trauma.

In summary, we demonstrated, for the first time, the efficacy of CPSI-121 in preventing intestinal barrier dysfunction similar to that seen after VNS. These preclinical animal studies demonstrate that a new pharmacologic agent given during trauma resuscitation can be successful. We think that this new data presented here should lead to further study focusing on the anti-inflammatory effects of CPSI-121 as a potential vagomimetic drug. We plan to test this vagomimetic candidate in more severe models of burn injury with greater percentage of TBSA burned and in trauma/hemorrhagic shock. This could have major implications for the treatment of the severely injured patient, which could limit posttraumatic sterile inflammatory responses and improve outcomes.

Biography

DISCUSSION

Dr. Basil A. Pruitt, Jr. (San Antonio, Texas) – Dr. Krzyzaniak, Dr. Coimbra and their colleagues have extended their studies of the effect of the parasympathetic nervous system on enterocyte morphology and barrier function following burn injury by assessing the vagomimetic effects of the infusion of the guanylylhydrazone-derived compound CPSI-121.

Apart from vagomimetic effects, guanylylhydrazone derivatives have derivative-specific effects e.g. the derivatives of pyridine-2 and pyridine-4 aldehydes inhibit thromboxane B2 biosynthesis. The pyridine-2 derivative selectively inhibits thromboxane synthase in association with a concomitant increase in prostaglandin E2 (PGE2). The pyridine-4 derivative, which is more active, markedly decreases PGE2 and inhibits cyclooxygenase. Consequently, we need to know the derivation of CPSI-121 and more about its systemic effects and effects on thromboxane synthesis. Additionally, the effect of methylglyoxal bis (guanylylhydrazone) on RNA polymerase activity in rat liver nuclei appears to be concentration dependent, i.e. stimulating enzyme activity below 3 mM and inhibiting activity above 3 mM. Have you performed dose-ranging studies to determine whether the activity of CPSI-121 is concentration dependent?

Recent studies by investigators in Wuhan, China have shown, in a murine model of burn injury, that vagal nerve stimulation increased mean arterial pressure and heart rate, decreased inflammatory cell infiltration of pulmonary interstitial and alveolar spaces, and

attenuated TNF α and IL-6 production. Do the effects of CPSI-121, which you report, represent a direct effect on intestinal permeability or the effects of increased tissue blood flow, a reduction in neutrophil infiltration, and lesser production of inflammatory cytokines?

Since semapimod (CNI-1493) is known to suppress inflammatory cytokines and systemic inflammation by direct activation of the vagus nerve, why did you choose to evaluate CPSI-121 and how do its systemic physiologic actions differ from those of CNI-1493? Specifically, what was the hemo-dynamic response to CPSI-121 in terms of small bowel blood flow and interluminal pressure in the isolated segment of bowel used for the permeability studies?

Figures 2 and 3 appear to indicate that splenectomy, without vagal stimulation or CPSI-121, has the same effect on gut injury score and intestinal permeability as the other interventions. Are those figures mislabeled or does that indicate a major role for inflammatory cells of splenic origin in the response to burn injury?

Lastly, in light of your earlier studies, was the effect of CPSI-121 on myosin light chain kinase comparable to that of vagal stimulation?

Finally, as you know, Dr. Cioffi asked me to be the invited discussant of your paper because in 1982 I underwent surgery that included a truncal vagotomy for a bleeding peptic ulcer. I had hopes that you had found a way to protect in my “vagally unnerved” state but, alas, the beneficial effect of CPSI-121 depends on an intact vagus nerve. In light of that and if your figures are correctly labeled to indicate a protective effect of splenectomy, do you and Dr. Coimbra recommend that I undergo prophylactic splenectomy to protect me from post-injury gut dysfunction?

Dr. Erik Barquist (Miami, Florida): I have two methodologic questions: The first, why did you choose to perform abdominal vagotomy rather than a cervical vagotomy, thereby keeping the abdomen pristine?

And, secondly, to echo Dr. Pruitt's question, why did you choose in this fundamentally mechanistic study to use a new third generation guanylhydrazone as opposed to one of the older agents? Thank you for your nice study.

Dr. Michael Krzyzaniak (San Diego, California): Thank you very much for your questions, Dr. Pruitt, and for your discussion of our paper.

To clarify one question off the top, the animals that received splenectomy did, in fact, receive the drug after the burn insult so I will definitely double check those figures. However, when administered, the drug compound seemed to work independent of the spleen.

Another one of your questions was related to dose dependency and effects. Prior to performing this particular study, we did perform dose-response studies to determine what dose would be effective for these mice. In fact, we performed 100-fold dilutions of the drug

compound and found, consistently, that the one milligram per kilogram dose was effective in preventing gut injury.

Additionally, we also looked at some elimination effects and found that at four hours after injury, which is the time point that we performed all of our assays, there was detectable drug levels still remaining in the serum of these animals.

You asked a question about the direct effects of CPSI-121 on intestinal permeability and specifically blood flow in the area of where the permeability was performed. We did not specifically measure blood flow in that area. Technically, that would be too difficult to do in the mouse.

We do know, however, from other studies that we've performed specific to trauma and hemorrhagic shock models that vagus nerve stimulation does not disrupt or change blood pressure or MAP in respect to how these animals compensate for their injury.

You asked specifically the differences between CNI-1493 and CPSI-121. And really the difference is that CNI-1493 is a tetravalent and guanylylhydrazone, the new compound, CPSI-121, is a divalent guanylylhydrazone.

This is the first time that the CPSI-121 has been used in an animal study. As a result, more specific differences have yet to be determined. We chose to pursue a functional readout as our first approach for this compound.

Another question you asked was related to the mechanism of action of the drug and how it functions in relation to vagus nerve stimulation and reduction in inflammation.

The origin of the guanylylhydrazone compound was specifically targeted at reducing nitric oxide production from macrophages by inhibiting L-arginine transport across the membrane. A series of studies did show that L-arginine transport was affected, but also further studies have shown that even in the presence of low extracellular arginine there was a reduction in macrophage stimulation and cytokine production.

Taking that one step further, authors found that the effects of the guanylylhydrazone were independent of the NF-kappa-B pathway such that mRNA levels were not affected but the post-translation and secreted levels of TNF-alpha were where the difference appeared.

More recent experiments with this drug, at least with the older generation compound, have shown that it was a P-38 MAP kinase inhibitor; however, when it was administered intracerebroventricularly it had the effect of not only reducing local TNF-alpha in the brain but also systemic TNF-alpha. This finding really began to put us down the path of whether or not guanylylhydrazones interact with the vagus nerve and the anti-inflammatory cholinergic pathway. That's what led us to perform this study with the new generation, CPSI-121, after seeing what was done with the older generation compound.

In this set of experiments, we did not measure inflammatory cytokines produced either systemically or in the gut, to include myosin light chain kinase. Looking at those readouts is the subject of planned future experiments.

Lastly, we have looked at the clinical effects of vagotomy in trauma outcomes. In a recently published paper through the Archives of Surgery, taking into account all of the limitations of a retrospective review of data, we found that disruption of the neuro-enteric axis by vagotomy does lead to worse outcomes in human trauma patients.

Dr. Barquist asked why we performed an abdominal vagotomy as opposed to a cervical vagotomy. The abdominal vagotomy was a method that we had done in the past with good success that was easy and feasible at the time. It would have been very difficult in a mouse to locate the cervical vagus nerve, perform a vagotomy there, and then be able to stimulate the distal end of that vagus nerve. So for technical reasons we performed the abdominal vagotomy as opposed to the cervical.

And the last question from Dr. Barquist about why we used the new generation compound as opposed to the old generation was simply a matter of availability. The old generation compound is currently in Phase III clinical trials for Crohn's Disease and Cytokine PharmaSciences offered this new compound to us and we took advantage of it.

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Figure 1.

Intestinal histology at 4 hours after burn. VNS and CPSI-121 equally protect against burn-induced intestinal injury, whereas vagotomy eliminates such protection. Sections of distal ileum were harvested 4 hours after 30% TBSA burn (n = 3 animals per group) and stained with hematoxylin and eosin. All images are taken at $\times 20$ magnification with black bar = 60 μm . (A) Sham animal and (B) sham with CPSI-121 treatment. Both images demonstrate an uninjured pattern. (C) Burn-induced injury marked by villus tip blunting, tip necrosis, and paucity of full-length villi. (D and E) CPSI-121 and VNS protect against intestinal injury after burn. (F) CPSI-121 protects against intestinal injury in the absence of the spleen. (G and H) Surgical abdominal vagotomy eliminates the protection offered by VNS and CPSI-121.

Figure 2.

Intestinal permeability to 4-kDa FITC-dextran 4 hours after burn. CPSI-121 and VNS similarly protected the intestine from an increase in permeability after burn, whereas abdominal vagotomy eliminates such protection. The absence of the spleen did not affect the protection by CPSI-121. * $p < 0.05$ vs. burn only; # $p < 0.05$ vs. vagotomy/ burn + VNS; + $p < 0.05$ vs. vagotomy/burn + CPSI-121 (n = 5 animals per group).

Figure 3.

Intestinal ZO-1 immunofluorescence at 4 hours after burn. (*A* and *B*) When viewed under confocal microscopy, tight cell-cell coaptation is readily evident in sham and animals receiving only CPSI-121. (*C*, *arrows*) After burn injury, gaps between cells are seen. (*D* and *E*) CPSI-121 and VNS similarly prevented the separation between cells. (*F*) In the absence of the spleen, CPSI-121 maintained the tight junctions. (*G* and *H*, *arrows*) Abdominal vagotomy performed before burn with either VNS or CPSI-121 administration eliminates the protective effect and paracellular gaps are readily apparent. $n = 4$ animals per group; size bar = 10 μm .

Figure 4.

Paracellular space measurements between intestinal epithelial cells at 4 hours after burn. VNS and CPSI-121 similarly prevented the increase in intercellular distance after burn. CPSI-121 also maintained the tight junctions after splenectomy. Abdominal vagotomy eliminated the protective effect of VNS and CPSI-121 and produced intercellular spaces similar to burn alone. * $p < 0.05$ vs. burn only; # $p < 0.05$ vs. vagotomy/burn + VNS; + $p < 0.05$ vs. vagotomy/ burn + CPSI-121 (n = 4 animals per group).

Figure 5.

Intestinal occludin immunofluorescence at 4 hours after burn. Exposure-matched images demonstrated preservation of intestinal epithelial occludin expression with VNS and CPSI-121. (A and B) Unburned animals without and with CPSI-121 produced strong staining of the tight junction protein, occludin. (C, arrow) Burn insult led to reduction in protein expression. (D and E) CPSI-121 and VNS after burn preserved higher concentrations of protein expression at the cell periphery. (F) Splenectomy did not affect the ability of CPSI-121 to maintain occludin expression. (G and H, arrows) Abdominal vagotomy eliminated the protective effect of VNS and CPSI-121, which led to reduced occludin expression after burn. n = 4 animals per group; size bar = 10 μ m.

Figure 6.

Occludin immunoblot of gut lysates at 4 hours after burn. Representative Western blot for the occludin protein is shown with its corresponding beta actin loading control to demonstrate equal protein load in all lanes. VNS and CPSI-121 resulted in preservation of protein expression when performed on animals with an intact neuroenteric axis. Significant reduction in occludin expression was seen after burn and if abdominal vagotomy was performed. * $p < 0.05$ vs. burn; # $p < 0.05$ vs. vagotomy/burn + VNS; + $p < 0.05$ vs. vagotomy/burn + CPSI-121 (n = 5 animals per group).