Published in final edited form as:

J Surg Res. 2010 September ; 163(1): 102–109. doi:10.1016/j.jss.2010.02.030.

Evaluating the potential role of nitric oxide as a mediator of hydrostatic edema mediated intestinal contractile dysfunction

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Abstract

Background—Administration of L-nit, a selective inhibitor of inducible nitric oxide synthase (iNOS), improves ileus in an animal model of resuscitation induced intestinal edema. The purpose of this study was to elucidate the iNOS/nitric oxide (NO) signal transduction pathway in intestinal edema.

Materials and Methods—Male Sprague Dawley rats were divided into two groups; CONTROL and RESUS + VH (edema, 80cc/kg normal saline (resuscitation) with mesenteric venous hypertension). iNOS mRNA and protein, iNOS activity, NO tissue levels, soluble guanylyl cyclase (sGC) expression, and cyclic guanosine monophosphate (cGMP) levels were measured. As a functional endpoint, we evaluated intestinal contractile strength and frequency in L-nit treated animals.

Results—Edema was associated with increased iNOS mRNA and protein expression without subsequent increases in iNOS activity or tissue NO levels. There was no significant change in sGC expression or increase in cGMP induced by edema. Administration of L-nit did not decrease edema development or preserve contractile strength, but increased contractile frequency.

Conclusion—Hydrostatic intestinal edema is not associated with increased iNOS activity or tissue NO levels. Administration of L-nit in edema increases intestinal contractile frequency. This may represent a potential mechanism for the amelioration of ileus seen with the administration of L-nit.

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Conflict Statement: There are no known conflicts between the authors and the information presented in this paper.

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MeSH Keywords

Edema; Trauma; Resuscitation; Intestines; Nitric oxide; Ileus

Introduction

High volume resuscitation and damage control surgery strategies lead to alterations in hydrostatic and oncotic pressures resulting in the development of intestinal edema. Intestinal edema results in ileus, which leads to delayed enteral feeding, increased morbidity and increased healthcare costs. (1–5)

In an animal model, intestinal edema in the absence of classic ischemic or inflammatory changes leads to ileus. (6–13) We have shown edema induced ileus to be mediated by several factors, including early and sustained alterations in the mechanical properties of the intestine (i.e., stress, strain, filamentous to globular actin ratio, and changes in calponin and vimentin levels), activation of signal transduction and activator of transcription (STAT) 3, and decreased phosphorylation of myosin light chain₂₀ (MLC) leading to decreased intestinal contractility. Pharmacologic inhibition of STAT-3 improves intestinal contractility and ileus. (8, 11–13)

STAT-3 is a nuclear transcription factor that up-regulates inducible nitric oxide synthase (iNOS) gene expression. In models of disease, including post-operative ileus, inflammatory bowel disease, and hemorrhagic shock, iNOS has been implicated to mediate decreased intestinal contractility leading to ileus. (14–18) iNOS is an enzyme that synthesizes citrulline and nitric oxide (NO) from arginine. NO binds to soluble guanylyl cyclase (sGC) and increases cyclic guanosine monophosphate (cGMP) production by converting guanosine triphosphate (GTP) to cGMP. cGMP dependent protein kinases mediate (at least partially) decreased phosphorylation of MLC by activating MLC phosphatase. (19) An alternative and/or additive mechanism may involve hyperpolarization of cell membranes, inhibiting calcium release and decreasing intestinal contractility. (20, 21) Additionally, NO may inhibit certain neurotransmitters, including substance P and/or acetylcholine. (20, 21)

Moore-Olufemi et al. initially demonstrated that hydrostatic edema was associated with increased iNOS protein in the small intestine and pretreatment of rats with L-nit (a known selective inhibitor of iNOS) resulted in improved intestinal transit. (6) However, the mechanism for this was not entirely clear, and the iNOS/NO pathway in hydrostatic intestinal edema has not been fully elucidated. We hypothesize that iNOS mediates, at least partially, decreased intestinal contractility in edema through its effect on cyclic GMP. We sought to interrogate the major components of the canonical iNOS/NO signal transduction pathway by determining the pattern of iNOS gene expression, localizing it to the mucosa or muscular layers of the small intestine, and subsequently determining the effect of hydrostatic edema on tissue iNOS activity, NO levels, sGC expression and cGMP levels. Additionally, as a functional end point, we sought to determine whether the improvement in intestinal transit seen with administration of L-nit was secondary to an effect on intestinal contractile strength or frequency.

Materials and Methods

Intestinal Edema Model

All procedures were approved by the University of Texas Animal Welfare Committee and were consistent with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (HSC-AWC-07-141).

Male Sprague Dawley rats (250g – 370g) were fasted 12–16 hours prior to surgery with free access to water. The rats were anesthetized with isoflurane, and a fluid filled external jugular vein catheter was placed for administration of high volume crystalloids. A midline laparotomy was performed. The superior mesenteric vein (SMV) was dissected free from its mesenteric attachments without significant manipulation of the small bowel. Intestinal edema was induced as previously published and is described briefly below. (7)

Rats were randomized into two groups (Table 1); CONTROL and RESUS + VH (mesenteric venous hypertension (explained below) with 80 cc/kg of 0.9% normal saline; edema group). Additionally, four additional groups were performed to determine the functional effects of L-nil administration; CONTROL with vehicle (0.9% normal saline, VEH), CONTROL with L-nil, RESUS + VH + VEH, and RESUS + VH + L-nil. One hour prior to surgery, rats in the L-nil groups received 10mg/kg of L-nil (Product I-1001, AG Scientific, San Diego, CA) or corresponding amount of saline vehicle (VEH) via intraperitoneal injection (as done in our previous study). (6)

The control group rats had the laparotomy wound closed. Mesenteric venous hypertension was induced in the edema (RESUS+VH) groups by placing a 4-0 silk suture around the superior mesenteric vein and then tying the suture over a piece of PE-10 sized tubing. The PE-10 tubing was then removed, creating a non-occlusive outflow stricture which causes a sustained elevation in mesenteric venous pressure. By tying the suture over PE-10 sized tubing and subsequently removing the PE-10 tube after the knot is tied down, we ensure that the superior mesenteric vein is not completely occluded. We have shown previously that this elevates the mesenteric venous pressure into the clinically relevant range, corresponding to intra-abdominal pressures in patients undergoing high volume crystalloid resuscitation. (22) This was immediately followed by intravenous infusion of crystalloid (0.9% normal saline) slowly over five minutes. The laparotomy wound was then closed and external jugular vein catheter was removed. Rats were sacrificed at either 30 minutes, 2 hours, and 6 hours after closure of abdomen and emergence from anesthesia. The animals had free access to water from the end of surgery until time of sacrifice; however, they were not fed during this time. The number of rats per group is detailed in Table 1.

Measurement of Wet to Dry Ratios

To confirm development of significant intestinal edema, wet to dry ratios were measured at the 6 hour time point. After gently flushing the intraluminal contents of the proximal small bowel, the bowel was gently milked dry and its wet weight was immediately measured. The small bowel was subsequently placed in a 60°C oven and allowed to dry over 2–3 days, until the dry weight was constant. Tissue water was then determined from the following equation.

$$\text{Wet to Dry Ratio} = ([\text{wet weight} - \text{dry weight}] / \text{dry weight}) \quad \text{Equation 1}$$

RNA extraction and Quantitative Reverse Transcriptase – Polymerase Chain Reaction

RNA was isolated from frozen mucosal (for iNOS expression) and seromuscular (for iNOS and sGC expression) tissue samples using RNA-Bee (Tel-Test, Inc., Friendswood, TX) following the manufacturer's protocol. RNA samples were treated with an RNase inhibitor after isolation. Additionally, the samples were treated with DNase immediately after RNA isolation to remove genomic DNA contamination.

Specific quantitative assays for iNOS, sGCα1, sGCβ1 and 36b4 were developed using Beacon Designer, AlleleID (Premier Biosoft), or RealTimeDesign (Biosearch Technologies) based on the NCBI refseq sequences. Assay information is provided in Table 2.

cDNA was synthesized by the addition of reverse transcriptase (RT) master mix (400 nM assay-specific reverse primer, 500 μ M deoxynucleotides, Superscript II buffer and 1 U/ μ l Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA)) to a 384-well plate and followed by addition of sample RNA. Each sample was assayed in triplicate plus a control without RT to access for DNA contamination. Each plate also contained an assay-specific synthetic amplicon oligo (sDNA) standard spanning a 5-log template concentration range and a no template PCR control. Each plate was covered and incubated for 30 minutes at 50°C followed by 72°C for 10 min.

Polymerase chain reaction (PCR) master mix was added directly to the RT volume. Final concentrations for the PCR were 400 nM forward and reverse primers (IDT, Coralville, IA), 100 nM fluorogenic probe (Biosearch Technologies, Novato, CA), 5 mM MgCl₂, and 200 μ M deoxynucleotides, PCR buffer, 150 nM SuperROX dye (Biosearch Technologies, Novato, CA) and 0.25 U JumpStart Taq polymerase per reaction (Invitrogen, Carlsbad, CA). Each plate was then covered and run using the following cycling conditions: 95°C, 1 min; followed by 40 cycles of 95°C, 12 sec and 60°C, 30 sec.

Synthetic, PAGE purified DNA oligos used as standards (sDNA) encompassed at least the entire 5' – 3' amplicon for the assay (Sigma-Genosys, The Woodlands, TX). Each oligo standard was diluted in 100 ng/ μ l *E. coli* tRNA-H₂O (Roche Diagnostics, Indianapolis, IN) and spanned a 5-log range in 10-fold decrements starting at 0.8 pg/reaction.

The final data for iNOS were normalized to 36B4 (ribosomal phosphoprotein P0). The final data for sCGa1 and sCGb1 were normalized to cyclophilin (peptidylprolyl isomerase A). The final data are presented as the molecules of unknown transcript/molecules of normalizer transcript and expressed as a fraction of normalizer transcript.

iNOS Immunofluorescence

Full thickness tissue segments from the distal small bowel were obtained from CONTROL and RESUS + VH groups sacrificed at the 6 hour time point. The tissues were fixed in buffered 10% formalin for at least 24 hours. After embedding in paraffin, 7 μ m sections were placed onto slides. After deparaffinization and antigen recovery, slides were incubated with a primary iNOS antibody (1:1000 dilution, Cayman Chemical, Ann Arbor, MI) and appropriate phycoerythrin conjugated fluorescent secondary antibody (1:200 dilution, Abcam, Cambridge, MA). The slides were then stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) for nuclear visualization and 2D deconvolution microscopy was utilized to determine iNOS localization. Relative intensity of staining for iNOS was determined by mean pixel count analysis (Corel Draw, Corel, USA).

iNOS/cNOS Activity Assay

Approximately 6 hours after surgery, segments of ileum were removed and had the mucosal layer scraped off. The tissue samples were immediately frozen in liquid nitrogen and stored at –80°C until ready for use. iNOS activity (as indicated by the conversion of radiolabeled (³H) arginine to citrulline) was measured in seromuscular tissue extracts utilizing a commercially available kit as per manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). We additionally determined constitutional NOS (cNOS) activity utilizing the same commercially available kit and reaction, except in the presence of calcium and calmodulin. All samples were assayed in duplicate and normalized to total protein concentration (as determined by the Quick Start Bradford protein assay, Bio Rad, Hercules, CA).

Tissue NO Levels

Approximately 6 hours after surgery, segments of ileum (approximately 0.5 inches long) were removed and had the mucosal layer scraped off. The tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until ready for use. NO levels (measured as total nitrite level) were measured in seromuscular tissue extracts utilizing a commercially available nitrate/nitrite fluorometric assay kit as per manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). All samples were assayed in duplicate.

cGMP Levels

Approximately 6 hours after surgery, segments of ileum (approximately 0.5 inches long) were removed and had their mucosal layer scraped off. These segments were then equilibrated in Krebs-Ringer solution (103mM NaCl, 4.7mM KCl, 2.5mM CaCl_2 , 25mM NaHCO_3 , 1.1mM NaH_2PO_4 , and 15mM glucose) gassed with 5% CO_2 -95% O_2 for approximately 45 minutes with 3 changes of solution. The segments were then incubated in 1mL of Krebs-Ringer solution with 0.3mM 3-isobutyl-1-methylxanthine (IBMX, a nonspecific phosphodiesterase inhibitor) and vehicle (0.1% dimethylsulfoxide) for 10 minutes. The tissues were then removed from solution and immediately frozen in liquid nitrogen and stored at -80°C . The tissue pieces were then ground with a mortar and pestle over liquid nitrogen and extracted by adding 300 μL of 1M perchloric acid (HClO_4). The samples were incubated on ice for 1 hour and then centrifuged at $10000 \times g$ for 5 minutes at 4°C . The supernatant was neutralized with 2M K_2CO_3 . The pellet was removed and the supernatant frozen (-20°C) overnight. After repeat centrifugation ($10000 \times g$ for 5 minutes at 4°C), the supernatant was used for an acetylated cGMP EILSA per manufacturer's suggested protocol (Cayman Chemical, Ann Arbor, MI). Samples were assayed in duplicate and normalized to tissue weight.

Measurement of Intestinal Contractile Strength and Contractile Frequency

Contractile activity was measured in the distal small intestine at 6 hours in CONTROL + VEH, CONTROL + L-nil, RESUS + VH + VEH, and RESUS + VH + L-nil groups according to methods which we have previously published. (12) Briefly, whole thickness strips were mounted in 25 mL organ baths filled with Krebs-Ringer solution (103mM NaCl, 4.7mM KCl, 2.5mM CaCl_2 , 25mM NaHCO_3 , 1.1mM NaH_2PO_4 , and 15mM glucose). The strips were mounted in duplicate. The solution was buffered with albumin to avoid edema formation during incubation in the tissue chamber and gassed with 5% CO_2 -95% O_2 . Isometric force was monitored by an external force displacement transducer (Quantametrics, Newtown, PA) connected to a PowerLab data acquisition system (AD Instruments, Colorado Springs, CO). Each strip was stretched to optimal length then allowed to equilibrate for at least 30 min. After equilibration, 30 min of basal contractile activity data (contractile strength and frequency) was recorded.

After recording contractile activity, the strip of intestine was removed, blotted lightly, and weighed. The length of each strip was measured. The cross-sectional area of each strip was calculated from length and weight data by assuming that the density of smooth muscle was 1.05 g/cm^3 . All force development was normalized to tissue cross-sectional area and expressed as stress ($\text{g/cm}^2/\text{s}$). Contractile activity was calculated as the area under the curve for 5 minutes. Contractile frequency was measured over the same time period (Hz).

Statistics

Unless otherwise indicated, all values are represented as mean $\pm$ SEM. Values were compared using one and two way analysis of variance (ANOVA). A p value of ≤ 0.05 was used to denote statistical significance.

Results

Wet to Dry Ratios

Mesenteric venous hypertension combined with high volume crystalloid resuscitation resulted in development of significant intestinal edema at six hours (3.8 ± 0.06 versus 3.3 ± 0.06). Edema was not ameliorated by administration of L-nil. After 6 hours, there was a significantly higher wet to dry ratio in the RESUS + VH + VEH group (3.77 ± 0.09) and RESUS + VH + L-nil (3.75 ± 0.06) groups versus CONTROL + VEH (3.22 ± 0.06) and CONTROL + L-nil (3.22 ± 0.02). Values are expressed as a unit less ratio.

iNOS mRNA Expression

Edema induced by high volume crystalloid resuscitation and mesenteric venous hypertension caused an approximately 4 fold increase in iNOS mRNA expression in the seromuscular layer at 6 hours (2 ± 0.6 versus 10 ± 3 in CONTROL and RESUS + VH groups, respectively), as determined by qPCR. (Figure 1a) There was no significant increase in iNOS expression at 30 minutes or 2 hours. There was no significant difference in iNOS expression between the CONTROL and RESUS + VH groups in the mucosal layer at 30 minutes, 2 hours or 6 hours. (Figure 1b) Values are expressed as iNOS/36b4 mRNA.

iNOS Immunohistochemistry

iNOS was significantly up-regulated in edematous intestine at 6 hours, as determined by mean pixel count analysis. (CONTROL (5.92 ± 1.03) versus RESUS + VH (17.68 ± 0.92)) Representative images (10x) are shown in Figure 2. Although there appears to be some iNOS staining in the peri-muscularis region, the up-regulation appears to be diffuse. Values are expressed as relative units.

iNOS/cNOS Activity

At 6 hours, edema did not increase iNOS activity in the seromuscular layer as compared to CONTROL (221.1 ± 28.8 versus 434.9 ± 91.6). Values are expressed as iNOS activity per microgram protein. There was no detectable cNOS activity in edematous tissues.

Tissue NO Levels

At 6 hours, there was no significant difference in seromuscular NO levels in RESUS + VH versus CONTROL (2.11 ± 0.35 versus 2.71 ± 0.27). Values are expressed as total micromolar nitrite.

sGC Expression

Seromuscular sGC α 1 expression (at 6 hours) was not significantly different in RESUS+VH as compared to CONTROL (1.41 ± 0.25 versus 3.23 ± 1.02 ; 2.3 fold). sGC β 1 expression was significantly reduced in RESUS + VH as compared to CONTROL (3.69 ± 0.45 versus 5.5 ± 0.69 ; 1.5 fold). Values are expressed as sGC α 1/cyclophilin and sGC β 1/cyclophilin mRNA.

cGMP Levels

There was no statistical difference between basal cGMP levels (at 6 hours) in the seromuscular layer of RESUS+VH (0.09 ± 0.04) animals as compared to CONTROL (0.20 ± 0.08). Values are expressed as fmol per mg tissue.

Basal Intestinal Contractile Strength and Frequency

Edema significantly reduced basal intestinal contractile strength. This was not reversed with administration of L-nil. At 6 hours, contractile strength was significantly higher in CONTROL + VEH (4.9 ± 1.4) and CONTROL + L-nil (6.2 ± 1.9) versus RESUS + VH + VEH (1.3 ± 0.26) and RESUS + VH + L-nil (0.7 ± 0.08). (Figure 3a) Values are represented as g/cm²/s. There was no differences in basal intestinal frequency between CONTROL + VEH (0.22 ± 0.03), CONTROL + L-nil (0.23 ± 0.03), and RESUS + VH + VEH (0.19 ± 0.07). However, administration of L-nil in rats subjected to mesenteric venous hypertension and high volume crystalloid resuscitation resulted in a significant increase in basal intestinal contractile frequency (0.53 ± 0.06). (Figure 3b) Values are represented as Hz.

Discussion

iNOS does not appear to be an early mediator of hydrostatic edema-induced intestinal contractile dysfunction. We demonstrate that, at 6 hours, hydrostatic intestinal edema was not associated with increases in iNOS activity, tissue NO levels, sGC expression or cGMP levels. While the data presented in this paper is generally considered “negative” data, the results are important for several reasons. In previously published work, we have taken a reductionist approach to the study of intestinal edema mediated dysfunction, i.e., studying the effects of intestinal edema in a setting free of classic ischemia, ischemia/reperfusion, and inflammatory factors. Our global hypothesis is that edema serves as a signal initiator and/or amplifier of dysfunctional signaling pathways. We have been able to reproduce alternations in signal transduction cascades historically described in models of global/regional ischemia/reperfusion and inflammation via induction of edema alone, as we have demonstrated with the transcription factor STAT-3 as well as other components of the intestinal contractile pathway. Although in previous work we have demonstrated that edema is associated with many of the same classic findings of dysfunction in other trauma models, edema does not appear to be the initiator for iNOS activation at the 6 hour time point.

We have historically measured intestinal contractility and transit at the six hour time point to exclude potential effects of anesthesia and/or laparotomy. We initially performed a time course experiment to determine the pattern of change in iNOS transcript levels. At six hours, intestinal edema is associated with increased iNOS protein (6) and increased iNOS expression in the seromuscular layer. It is interesting to note that while transcript levels are elevated, the increase is lower than has been reported in other models in which ileus is a component, including hemorrhagic shock and bowel manipulation. (17, 23) At this time point, we did not find an associated increase in iNOS activity, NO levels, sGC expression, or cGMP levels. We initially were interested in the role of iNOS secondary to the positive effects of L-nil in intestinal edema. Elucidation of all of the major components of the iNOS/NO pathway appears to strengthen the assertion that the mechanism of L-nil in improved intestinal transit at the six hour time point is independent of iNOS activity or cGMP levels.

Although L-nil is classically described as a selective inhibitor of iNOS (28 times more selective), it may have an effect on constitutive NOS (cNOS), especially at the dose that we and others have used in this and previous studies. (6, 24, 25) To exclude a potential role of cNOS in hydrostatic intestinal edema, we evaluated the effect of our edema model on cNOS activity. cNOS activity was not detected in edematous intestinal seromuscular tissue (not significantly different from control animals), suggesting that any potential mechanism of L-nil in edema is likely not secondary to an effect on cNOS.

There is little published in the literature regarding non-specific effects of L-nil. (26) Previous microarray analysis of L-nil treated edema rats revealed that L-nil pre-treatment prevented the change in a variety of signal transduction genes, including STAT-3. (27) We

have shown previously that STAT-3 at least partially mediates ileus in hydrostatic intestinal edema. Pharmacological inhibition of STAT-3 results in partial improvement of intestinal contractility and ileus. (13) We have shown that STAT-3 nuclear activation is decreased in L-nil treated RESUS + VH animals. (DATA NOT SHOWN) However, given the fact that we do not note an improvement in intestinal contractile strength with the observed decrease in STAT-3 nuclear activation leads us to believe that the mechanism of L-nil is likely not mediated by an effect on STAT-3.

L-nil has been shown to affect STAT-3 activation in other models (e.g., hemorrhagic shock); however the effect is believed to be secondary to its effect on NO production. (28) iNOS and NO are widely implicated in models of intestinal pathology in which a common factor includes inflammatory or ischemia-related injury, including hemorrhagic shock and resuscitation, ischemia/reperfusion, peritonitis, and post-surgical ileus. (14, 18, 25, 29, 30) In addition to its effect on myosin light chain phosphatase, iNOS is also believed to contribute to decreased intestinal contractility secondary to promotion of neutrophil infiltration into the muscularis as well as increasing certain pro-inflammatory cytokines (including IL-6, a known activator of STAT-3). (17) In our model of intestinal edema, there is no evidence of neutrophil infiltration or elevation of pro-inflammatory cytokines. (7, 12) Based on our published data, we suspect that STAT-3 elevation in our model occurs via a non-canonical pathway and not via an inflammatory related mechanism. (31–33) Additionally, somewhat different from other models, iNOS mRNA is elevated much later than STAT-3, suggesting it is not a preceding step. (13) iNOS/NO does not seem to be a major modulator or initiator of signaling cascades that cause ileus, specifically STAT-3, in hydrostatic edema.

The intestinal contractile complex is the obligatory step in the regulation of intestinal transit. Previously published research indicates that most therapeutic modalities that improve intestinal transit at least partially improve intestinal contractility. (13, 31) It is somewhat interesting, but not unexpected, that the iNOS inhibitor L-nil did not improve intestinal contractile strength in hydrostatic edema as edema was not associated with increased cGMP levels. However, we did note increased intestinal contractile frequency. Less powerful, but more frequent intestinal contractions could explain the improvement in intestinal transit seen with administration of L-nil. As has been suggested by other investigators in other disease models, the effect on intestinal frequency may be secondary to an effect of L-nil on the interstitial cells of Cajal and the enteric nervous system and is a subject of potential future investigation. (34)

iNOS is described as a constitutively active gene product. Therefore, the increase in iNOS expression and protein (by immunohistochemistry and western blot) at 6 hours with no change in iNOS activity may be somewhat puzzling. Similar to cNOS, increasing evidence suggests that post-translational modification, including phosphorylation appears to be necessary for increased iNOS activity. (35–37) Pan et. al. demonstrated that increases in nitrite (a surrogate marker of increased iNOS activity and NO production) followed increases in iNOS expression and protein by hours. (37) Other investigators have noted similar findings. (38, 39)

Given the fact that L-nil improves intestinal transit at 6 hours, it does not appear that an effect on iNOS represents the mechanism of benefit. The therapeutic effect of L-nil administration may be secondary to an effect on intestinal contractile frequency. However, it is important to note that the findings presented does not exclude the notion that iNOS may mediate later (i.e., after 6 hours) effects of intestinal edema.

Acknowledgments

The authors would like to acknowledge Gregory Shipley, PhD and the Quantitative Genomics Core Laboratory (QGCL) at The University of Texas Health Science Center at Houston for their assistance with RT-PCR.

SOURCES OF SUPPORT: NIH Grants T32 GM 0879201, KO1 DK 070758, RO1 HL 092916, and P50 GM 38529; Children's Memorial Hermann Hospital Foundation; University of Texas Office of Biotechnology Seed Grant

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Figure 1a:

Figure 1b:

Figure 1.

Figure 1a. iNOS expression is increased approximately 4 fold in the seromuscular layer of edematous intestine at 6 hours. Note that there is no increase in iNOS expression in the mucosa at any time point. * indicates statistical significance versus CONTROL.

Figure 2.

Immunohistochemistry indicates marked upregulation of iNOS in edematous as opposed to control sections of intestine (10x; Blue=DAPI, Red=iNOS). Scale bars represent 100 μm. Figure 2b. There is no significant increase in iNOS expression in the mucosal layer at 30 minutes, 2 hours, or 6 hours.

Figure 3a:

Figure 3b:

Figure 3.

Figure 3a. Basal intestinal contractile strength is significantly reduced with development of edema; this is not ameliorated by administration of L-nil. * indicates statistical significance versus CONTROL.

Figure 3b. Basal intestinal contractile frequency is significantly increased in the RESUS + VH + L-nil group. * indicates statistical significance versus CONTROL and RESUS + VH + VEH.

Table 1

Experimental group setup

Experiment 1: Experiment Setup (Effect of Edema on iNOS Pathway)						
CONTROL			RESUS + VH			
30 minutes	2 hours	6 hours	30 minutes	2 hours	6 hours	
n=5	n=5	n=6	n=5	n=5	n=6	
Experiment 2: Functional Effects of L-nil						
CONTROL			RESUS + VH			
VEH	L-nil	VEH	L-nil			
n=4	n=4	n=4	n=4			

Table 2

Primers and probes utilized for qPCR analysis

Transcript	Accn. #	Forward Primer	Reverse Primer	Fluorogenic Probe	Amplicon Length	Lowest Limit of Detection	PCR Efficiency
36B4	NM_022402	AGAGGTGCTGGACATCACAG	CATTGCCGGACACCCTCTAG	FAM-CAGGCCCTGCACACTCGCTT-BHQ1	62	230	99%
iNOS	NM_012611	GAGAAAGCTGAGGCCCCAGG	ACCTTCCGCAATTAGCACAGA	FAM-CAGTCTTTGGTGAAAG CGGTGTTCTTTTG-BHQ1	86	170	95%
Cyclophilin	NM_017101	CTGATGGCGAGGCCCTTG	TCTGCTGTCTTTTGGAACTTTGTC	FAM-CGGGTCTGCTTCGAGCTGTTTGCA-BHQ1	67	220	96%
sGCa1	NM_346628	CGCTCTCTATACTCGCTTTTGACC	ACACAATATGCACTCTCCGATGG	FAM-CCACCTTTGTAGACATCCAGCTCTCCACA-BHQ1	80	180	97%
sGCb1	NM_012769	TCAGCGCAGTGTGGAAATGC	GCGGACCAGAGAGAACACAGA	FAM-ACAGAGTGTCTCCCCCAGCTCCAG-BHQ1	89	160	95%