Injury and Intestinal Barrier Dysfunction: Past, Present, and Future

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Department of Surgery
University of California San Diego School of Medicine
Background

• The intestine plays a significant role in the systemic inflammatory response (SIRS)

• SIRS can lead to distant organ injury, multi-organ failure, and death

• Our understanding of the gut’s role in causing SIRS has evolved over the past several decades
Bacterial Translocation

- **1980’s: Gut Origin of Sepsis**
  - Passage of luminal bacteria (endotoxin) into portal circulation
  - Bacteria found in mesenteric lymph nodes
  - Bacteria reaches systemic circulation via portal vein
    - Kupffer cells produce cytokines
  - Systemic Inflammatory Response

Bacterial Translocation

• Early 1990’s: Bacterial Translocation in question

• Moore, et al: Is there enteric bacteria in the portal blood of severely injured trauma patients?
  – 20 injured patients requiring emergent laparotomy
  – Portal vein catheters inserted
  – Blood drawn up to 5 days post-operatively
  – 8/212 (2%) of blood cultures positive
    • 7 presumed contaminants
    • 1 S. Aureus in patient with known S. Aureus pneumonia
  – Conclusion: No portal or systemic bacteremia despite 30% incidence of MOF in these patients

"We look hard at what is routinely done in the Shock Trauma ICU and ask, 'How does this treatment affect the gut function?' We are finding that when a person is critically ill, the gastrointestinal (GI) tract doesn't work. If we can make the gut work better, then we can prevent a lot of infection,"
• 1990’s-Present: Gut Inflammation
  – Gut barrier breakdown causes intestinal inflammatory response
    • Intestinal cytokine production
  – Gut-derived inflammatory mediators carried in intestinal lymph
  – Activated intestinal lymph causes SIRS, distant organ injury

1. Deitch, EA. Surgery. 2002;131:241-244
TRAUMA–SHOCK

- Decreased intestinal blood flow and altered intestinal permeability
- Bacterial translocation
- Systemic infection
  - Septic state
  - Increased portal endotoxemia
  - Systemic Endotoxemia
  - Distant organ failure (MODS)

SHOCK–TRAUMA–INDUCED DECREASE IN GUT BLOOD FLOW

- Gut ischemia–reperfusion injury
- Loss gut barrier function (Bacterial translocation)
- Gut–derived inflammatory factors carried in the mesenteric lymph
  - Septic state
  - Distant organ failure (MODS)
- Gut inflammatory response
Mesenteric lymph from burned animals:
- Activate PMNs
- Activate endothelial cells

Portal vein plasma did not activate PMNs

Intravenous Injection of Trauma-Hemorrhagic Shock Mesenteric Lymph Causes Lung Injury That Is Dependent Upon Activation of the Inducible Nitric Oxide Synthase Pathway

Maheswari Senthil, MD, Anthony Watkins, MD, Dimitrios Barkou, MD, Da-Zhong Xu, MD, PhD, Qi Lu, MD, Billy Abungu, BSc, Frank Caputo, MD, Rena Feinman, PhD, and Edwin A. Deitch, MD

Lymphatic Duct Ligation (LDL)
- Decreases histologic lung injury
- Decreases lung permeability
- Decreases neutrophil CD11b expression
Mesenteric lymph flow depends on depth of shock.

Maximal PMN priming by mesenteric lymph occurs in the 3rd hour post-shock.

Activity of mesenteric lymph depends on depth and duration of shock.

Arachidonic acid in postshock mesenteric lymph induces pulmonary synthesis of leukotriene B₄

Janeen R. Jordan,¹,² Ernest E. Moore,¹,² Eric L. Sarin,¹,² Sagar S. Damle,¹,² Sara B. Kashuk,¹ Christopher C. Silliman,¹,² and Anirban Banerjee³

**p<0.01

**p<0.01, vs preshock
*p<0.01, vs postshock
RINGER’S LACTATE

- Current standard resuscitation regimen
  - Potentiates neutrophil activation
    - Rhee et al. 1998
  - Contributes to end organ injury
    - Savage et al. 2005
Pentoxifylline (PTX)

- Non-specific Phosphodiesterase Inhibitor
  - Increases cyclic AMP
  - PKA activation

- Clinical Applications:
  - Intermittent Claudication
  - Alcoholic Hepatitis

- Animal Models:
  - Decreases pro-inflammatory cytokine activation
  - Attenuates neutrophil oxidative burst
  - Decreases distant organ injury
Hemorrhagic shock

Hemorrhagic Shock

Classic treatment
Ringer’s Lactate

Proposed treatment

Hemorrhagic Shock

Hypertonic Saline + Pentoxifylline

- Improves microcirculation
- Attenuates oxidative stress
- Downregulates neutrophil function
- Reduces host organ injury
Hemorrhagic Shock

Ringer’s Lactate (RL)
- Potentiate neutrophil activation
  *Resuscitation 2004*
- Promote endothelial dysfunction
  *J Trauma 2005*
- Contribute to end organ injury
  *J Trauma 2006*

HSPTX
- Reduce oxidative stress
- Downregulate PMN function
  *J Trauma 2005*
- Attenuate Post-shock Lung Injury
  *J Trauma 2006*
HSPTX Protects Against Hemorrhagic Shock Resuscitation-Induced Tissue Injury: An Attractive Alternative to Ringer’s Lactate

Raul Coimbra, MD, PhD, FACS, Rafael Porcides, MD, William Loomis, BS, Heidi Melbostad, BS, Rohan Lall, MD, Jessica Derre, MD, Paul Wolf, MD, and David B. Hoyt, MD, FACS

Sham

Ringers Lactate

HSPTX

Nitric oxide and Ischemia Reperfusion

- iNOS induction and production of sustained quantities of NO occur in the gut after I/R injury.

- Nitric oxide
  
  Direct effects on cell signaling: Transcription factor activation (NF-κB and STAT3) and cytokine production (TNF-α and IL-6)

  \[ J \text{ Exp Med 1998} \]

  Indirect cytotoxic effects: Peroxynitrite formation
Intestinal I/R Injury

\[ \downarrow \]

iNOS

\[ \downarrow \]

NO

\[ \downarrow \]

NF-κB/STAT3

\[ \downarrow \]

TNF-α, CINC, IL-6

\[ \rightarrow \]

Organ Injury

\[ \downarrow \]

Peroxynitrite
Hypothesis

• The attenuation in gut injury observed with HSPTX after hemorrhagic shock is associated with a decrease in intestinal iNOS activity and NO-mediated events including local pro-inflammatory cytokine production when compared to RL in vivo.
Methods

- **RL**: 32 mL/kg racemic RL (n=7)
- **HSPTX**: 4 mL/kg 7.5% NaCl + PTX 25 mg/kg (n=7)
- Sham group (n=5)
In this graph, the iNOS Content is shown for different groups: Sham, RL, and HSPTX. The Y-axis represents the ileal iNOS Content (Pixel Total + SEM) ranging from 0 to 120000. The X-axis lists the groups: Sham, RL, and HSPTX. Each group has a bar indicating their iNOS Content level with error bars showing the SEM. The red bar for RL reaches the highest level, indicating a significant difference compared to Sham and HSPTX. A asterisk (*) above the RL bar indicates a statistical significance at P < 0.05, suggesting a significant difference in iNOS Content compared to Sham and HSPTX groups.
Nitrite

* P < 0.05
Cytoplasmic I-κBα Phosphorylation

I-κBα Phosphorylation (Pixel Total + SEM)

Sham
RL
HSPTX

41 kD

* P < 0.01
Nuclear NF-κB Phosphorylation

NF-κB p65 Phosphorylation (Pixel Total + SEM)

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<thead>
<tr>
<th></th>
<th>Sham</th>
<th>RL</th>
<th>HSPTX</th>
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</thead>
<tbody>
<tr>
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<tr>
<td>SEM</td>
<td></td>
<td>20000</td>
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* P < 0.01

65 kD
STAT-3

[Image: Bar graph showing ileal STAT3 phosphorylation levels for Sham, RL, and RL+PTX conditions.]
TNF-α

Sham RL HSPTX

TNF-α Concentration (pg/mL + SEM)

* P < 0.01
Interleukin-6

IL-6 Concentration (pg/mL + SEM)

Sham  RL  HSPTX

* P < 0.01
Intestinal I/R Injury

\[ \text{iNOS} \]

\[ \text{NO} \]

\[ \text{NF-\kappa B} \]

\[ \text{Peroxynitrite} \]

\[ \text{TNF-\alpha, CINC, IL-6} \]

\[ \text{Organ Injury} \]
Hypertonic Saline and Pentoxifylline Attenuates Gut Injury After Hemorrhagic Shock: The Kinder, Gentler Resuscitation

Jessica Dere, MD, Tercio de Campos, MD, Edna Shervi, BS, William H. Loomis, BS, David B. Hoyt, MD, and Raul Coimbra, MD, PhD

Phosphodiesterase inhibition downregulates intestinal injury and inducible nitric oxide synthase activity after hemorrhagic shock

JESSICA DERE, WILLIAM H. LOOMIS, JAMES G. PUTNAM, PAUL WOLF, TODD COSTANTINI, DAVID B. HOYT and RAUL COIMBRA

TNF-α and Intestinal Barrier

The pivotal role of tumor necrosis factor-alpha in signaling apoptosis in intestinal epithelial cells under shock conditions.

Diebel LN, Liberati DM, Baylor AE 3rd, Brown WJ, Diglio CA

J Trauma. 2005 May;58(5):995-100
Gut Barrier Breakdown

• 2008: Intestinal Barrier Injury
  – Can we prevent the intestinal inflammatory response and subsequent SIRS by limiting intestinal barrier breakdown?

• Intestinal Tight Junction
  – Creates physical barrier that seals the space between adjacent epithelial cells
  – Regulates intestinal permeability
  – Modulation of tight junction proteins alters epithelial barrier function

Normal Intestinal Barrier

Table. Protective mechanisms of the intestine

<table>
<thead>
<tr>
<th>Mechanical</th>
<th>Nonmechanical</th>
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<tr>
<td>Peristalsis</td>
<td>Normal gut flora–mediated colonization resistance</td>
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<tr>
<td>Epithelial barrier</td>
<td>Secretory immunoglobulins</td>
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<tr>
<td>Mucus layer</td>
<td>Gut-associated lymphoid tissue</td>
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<tr>
<td>Tight junctions</td>
<td>Dendritic cells</td>
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<tr>
<td></td>
<td>Macrophages</td>
</tr>
<tr>
<td></td>
<td>Antigen receptors</td>
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Intestinal Tight Junction

• Occludin
  – Four transmembrane domains
  – Attaches adjacent cells at tight junction

• ZO-1
  – Attaches occludin to perijunctional actin cytoskeleton

• Myosin light chain kinase (MLCK)
  – Increases phosphorylation of myosin light chain (MLC)
  – Modulates contraction of the actin cytoskeleton
ZO-1
Occludin
Actin
TNF-α
IL1-β
IFN-γ

ZO-1
Occludin
Actin
TNF-α
IL1-β
IFN-γ
Caco-2 cells + Cytomix

Costantini T, et al., Life Sciences, 2009
Caco-2 cells + Cytomix

A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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<tbody>
<tr>
<td>ZO-1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-actin</td>
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225 kDa  
45 kDa

B

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<th>Cytomix</th>
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<tbody>
<tr>
<td>Relative Band Density (+/-SEM)</td>
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<td></td>
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</table>

Costantini T, et al., *Life Sciences*, 2009
Phosphodiesterase inhibition attenuates alterations to the tight junction proteins occludin and ZO-1 in immunostimulated Caco-2 intestinal monolayers

Todd W. Costantini, Jessica Deree, William Loomis, James G. Putnam, Sunghyuk Choi, Andrew Baird, Brian P. Eliceiri, Vishal Bansal, Raul Coimbra *

Life Sciences 84 (2009) 18–22
Burn-induced Histologic Gut Injury

Intestinal Occludin


* p < 0.01 vs. Sham
† p < 0.05 vs. Burn
‡ p < 0.01 vs. Sham
§ p < 0.05 vs. Burn
Intestinal ZO-1

6 hour

- **Sham**
- **Burn**
- **Burn/PTX**

24 hour

- **Sham**
- **Burn**
- **Burn/PTX**

**Relative Band Density (+/- SEM)**

* * p < 0.01 vs. Sham
† † p < 0.05 vs. Sham
‡ ‡ p < 0.05 vs. Burn

**ZO-1**

**β -actin**

Tight Junction Confocal Microscopy

BURN-INDUCED GUT BARRIER INJURY IS ATTENUATED BY PHOSPHODIESTERASE INHIBITION: EFFECTS ON TIGHT JUNCTION STRUCTURAL PROTEINS


Division of Trauma, Surgical Critical Care, and Burns, Department of Surgery, University of California-San Diego School of Medicine, San Diego, California

Intestinal Permeability

Costantini, et al.  *p < 0.001 vs. Sham
†p < 0.001 vs. Burn

UC San Diego Medical Center
Intestinal Barrier Breakdown

• Myosin light chain kinase (MLCK)
  – Increases phosphorylation of myosin light chain
  – TNF-α increases MLCK expression
  – Increased MLCK protein expression:
    • Decreases ZO-1 and occludin levels
    • Increases intestinal permeability

Intestinal Barrier Breakdown

- Intestinal NF-κB
  - NF-κB mediates activation of MLCK by binding to MLCK promoter
  - Inhibition of NF-κB p65 decreases MLCK activation

Ye, et al. Am J Physiol Gastrointest Liver Physiol 2006;290:496-504
Methods

30% TBSA steam burn for 7 seconds

IP injection:
12.5mg/kg PTX in 500 μl Normal Saline
vs.
500 μl Normal Saline

2hr

4hr

Harvest Distal Ileum:
Histology
TNF-α ELISA
Confocal Microscopy
- Phosphorylated MLC
Western blot
- MLCK
- Cytoplasmic IKK, IkBa
- Nuclear NF-κB p65

Intestinal Permeability:
4 kDa FITC-Dextran
Intestinal Myosin Light Chain Kinase

* p < 0.02 vs. Burn
Cytoplasmic Phosphorylated IKK$\alpha/\beta$

* $p < 0.05$ vs. Burn

Relative Band Density (+/- SEM)

- Sham
- Burn
- Burn/PTX

P-IKK$\alpha/\beta$

IKK$\alpha/\beta$
Cytoplasmic Phosphorylated IkBα

* p < 0.01 vs. Burn
Nuclear NF-κBp65

* p < 0.03 vs. Burn

Relative Band Density (+/-SEM)

- Sham
- Burn
- Burn/PTX

P-NF-κB p65
Beta-Laminin
Intestinal TNF-α

* p < 0.03 vs. Sham
† p < 0.05 vs. Burn

Intestinal TNF-α (pg/ml) ± SEM

Sham | Burn | Burn/PTX
Phosphorylated MLC Confocal Microscopy

Costantini, et al.  *J Trauma* 2009

Bar = 20 µm
Intestinal Permeability 4 Hours Post-Burn

![Graph showing FITC-Dextran levels in different groups: Sham, Burn, Burn/PTX. The burn group shows significantly higher FITC-Dextran levels compared to sham and burn/PTX.](image-url)
What is in the Future?
Future #1: Novel Imaging of Intestinal Injury

- Intraluminal placement of near-infrared dye
  - Alexa Fluor 680

- Imaging using Xenogen IVIS Lumina

- Quantification of fluorescence
  - Correlates with “classic” assays of intestinal injury and intestinal permeability
Near-infrared Imaging of Intestinal Injury

Sham  0hr Burn  4hr Burn  6hr Burn  24hr Burn  48hr Burn
Quantification of Near-infrared Imaging

**Abdominal Quantification**

- Sham: 5.0E+07
- 0hr: 4.0E+07
- 4hr: 3.0E+07
- 6hr: 2.0E+07
- 24hr: 1.0E+07
- 48hr: 0.0E+00

**Intestinal Quantification**

- Sham: 5.0E+08
- 0hr: 4.0E+08
- 4hr: 3.0E+08
- 6hr: 2.0E+08
- 24hr: 1.0E+08
- 48hr: 0.0E+00
Gavage Time Course

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<th>Min</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>60</th>
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<tr>
<td>4h Burn</td>
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Gavage Quantification

Sham 4h Burn

Abdominal Quantification

Fluorescent Intensity (±SEM)

Sham 4hr Burn

* p<0.03
Future #2

Utilizing Phage Display Technology to Identify Peptide Sequences Targeting the Burn Injured Intestinal Barrier

Todd W. Costantini MD, Carrie Y. Peterson MD, James G. Putnam BS, Ritsuko Sawada PhD, William H. Loomis BS, Brian P. Eliceiri PhD, Andrew Baird PhD, Vishal Bansal MD, Raul Coimbra, MD, PhD
Background

• Intestinal injury is known to result from several clinical conditions resulting in significant morbidity and mortality
  – Severe trauma, burn
  – Inflammatory bowel disease
  – Necrotizing enterocolitis

• The ability to effectively target the intestinal mucosa to deliver biotherapies could be of powerful clinical utility
  – Prevent gut injury
  – Speed intestinal barrier healing
Drug Delivery

• Delivery of therapeutics to the intestinal mucosa remains a difficult problem

• Must be delivered to the cells of the intestinal wall in sufficient quantities to achieve the desired effect
  – Issues of clearance
  – Timing of drug delivery
  – Alterations in perfusion to the gut following injury
Phage Display

- Used to identify functional targeting ligands and their corresponding receptors.

- Diverse libraries of peptide sequences ($1 \times 10^{12}$) can be displayed by utilizing the bacteriophage M13.

- Single peptide sequence is displayed on a single phage
  - Allows for biopanning of a large number of peptide sequences

Phage Display

- Phage-based vectors can be used to identify peptides which can perform targeted delivery of biotherapeutics
  - Genes, antibiotics, growth factors

- Screen for peptides that home to specific tissues

- Wide-ranging applications
  - Cancer Therapies:
    - Targeting tumor vasculature with TNF-α
    - Screening for antigens overexpressed by carcinomas

Hypothesis

• We postulated that by utilizing in vivo phage display, we would identify peptide sequences which internalize into the intestinal epithelial following severe injury

• We could bind this newly discovered peptide sequence to fluorescent nanoparticles in order to image its delivery into the gut barrier
Methods- Phage Screening

30% TBSA steam burn for 7 seconds

balb/c mice

2hr

• Intestinal mucosa isolated 2 hours following burn
• Mucosa incubated with Phage library containing $10^{12}$ different peptide sequence
• Selected Phage amplified using E. coli
• Process repeated 3 times to select for gut-targeting peptide sequence
Methods- Intraluminal Delivery of Phage

30% TBSA steam burn for 7 seconds

balb/c mice

2 hr

30 min

- Perform Laparotomy
- Isolate 3 segments of distal small intestine between silk ties
- Inject 200 μl containing 1 x 10⁹ phage or control (PBS or "empty phage")
- Close Abdomen

- Harvest each segment of distal small intestine
- Bowel segments washed with PBS, Trypsin using a peristaltic pump
- Phage DNA isolated from specimens for PCR
### Candidate Peptide Sequences

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<th>Peptide Sequence</th>
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<tr>
<td>YGFE LMVMASQV</td>
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<td>STYAVV TSMWWE</td>
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<tr>
<td>W LA PL PRM A I HT</td>
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- T-18 identified as candidate gut-targeting sequence
- Isolated in several rounds of screening
Ex Vivo Staining of Intestine

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<th></th>
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<th>AS8</th>
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<tr>
<td>anti-m13 + DAPI</td>
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Intestinal qPCR

Sham Animal

Particles per mg tissue

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<th>PBS</th>
<th>Empty Phage</th>
<th>T18-Phage</th>
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<tr>
<td>Count</td>
<td>6</td>
<td>4,451</td>
<td>38,591</td>
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</table>
Intestinal qPCR

2 Hour Burn

Particles per mg tissue

PBS  Empty Phage  T18-Phage

33  1,855  18,281
DNA Sequencing of PCR Product

12 mer

T18

5’- CGCAGAAGGC GGCGGCCAATCCT GAGGATGATAAG -3’ NS
3’- GCGTCTTGGCCGGCCTTACCCTACTCATTC -5’ S

mRNA  CTT  ACT  CAT  CCT  CAG  GAT  TCG  CCG  CCG  GCT  TCT  GCG
Protein  L  T  H  P  Q  D  S  P  P  A  S  A
Quantum Dots

• Fluorescent nanoparticles
• Emit light which can be visualized using confocal microscopy
• Peptide sequence coupled to Qdot
• Used as a reporter to visualize distribution of the peptide sequence
Qdot Imaging of T18 Sequence

Unconjugated Qdots

LYVE-1 (lymph)  Qdots  Overlay

Qdots - T18 Conjugate

Bar = 50µm
Summary

• Utilized phage display to screen for peptides that target the intestinal barrier

• Identification of a 12 amino acid peptide sequence that binds and internalizes into intestinal epithelial cells after burn injury

• Demonstrated delivery of fluorescent nanoparticles bound to the peptide sequence
Conclusion

- This sequence may allow for targeted therapies designed to attenuate intestinal dysfunction following severe injury, inflammation, or other pathologic conditions of the small bowel.
Future #3: The Neuro-Enteric Axis

• Enteric Nervous System
  – Gastrointestinal tissues innervated by complex component of the peripheral nervous system

• Enteric Glia
  – Similar to astrocytes of the CNS
  – Express glial fibrillary acidic protein (GFAP) when activated
  – Promote intestinal barrier function
    • Secretion of S-nitrosoglutathione (GSNO)
Mucosal barrier function depends on the integrity of intestinal epithelial cell (IEC) tight-junctions (A) which in turn rely on appropriate interactions with enteric glial cells (EGC), partly via release of *s*-nitrosoglutathione (B).

Savidge TC, et al. Lab Invest 2007;87:731-36
GFAP is required to maintain gut architecture

Sham vs. GFAP Conditional knockout

GFAP-HSV-Tk Mice
- Fatal by 19 days
- Severe inflammation
- Hemorrhagic necrosis

Inflammation activates enteric glia cells

Pro-inflammatory cytokines increase percentage of GFAP positive staining (red) neurons

Addition of enteric glia cells (EGC) to Caco2 cell culture:  
• Increases occludin and ZO-1 levels  
• Improves barrier function (TER and FITC-DexTRAN)
Enteric glia cells secrete GSNO when activated, which improves intestinal barrier function at low concentrations.

GSNO improves barrier function at low concentrations and increases permeability at high concentrations.
Intestinal GFAP qPCR Time Course

Fold Increase (+/-SEM)

- Sham
- 2 hour
- 6 hour
- 24 hour
Intestinal GFAP- Confocal Time Course

Green = S100
Red = GFAP
GFAP-luc Transgenic Mice

Sham

2hr

6hr

Burn

Gut

Brain
Quantification of Luminescence from GFAP-luc Mice

Luminescent Intensity (Arbitrary Units) ± SEM

- Sham
- 2hr Burn
- 8hr Burn

Luminescent intensity values are represented in arbitrary units.
Histology

- Sham
- 4h Burn
- Vagal Stim / Burn
- Vagotomy / Vagal Stim / Burn
Intestinal Permeability 4 hrs Post-burn

FITC-Dextran (ug/ml) +/- SEM

Sham: n=4
Burn: n=5
Cervical Vagal Stim + Burn: n=5
Abd Vagotomy + Burn: n=5
Cervical Vagal + Abd Vagotomy + Stim: n=5
Occludin Western blot 4hrs post-burn
Intestinal GFAP qPCR 4 hours post-burn

Gene Expression: GFAP in Burn Gene study_condition.gxd
GFAP Confocal - 4 hrs post-burn

- Sham (368)
- Burn (373)
- Burn + Vagal Stim (370)
- Burn + Vagotony + Vagal Stim (408)

60X Magnification Comparison

Villous Tips

Lamina Propria

Villous Tips

Lamina Propria
Conclusions

• Past: Translocation through the portal vein to liver.
• Present: Lymph route more important
• Future: Already here
  – Non-invasive method of monitoring organ injury. One animal – multiple measurements
  – Drug delivery to target cells. Specific, more effective, perhaps cheaper
  – Manipulation of PNS and enteric glia – promising therapeutic strategy.
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Thank you

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