Basic Study

**In vivo** analysis of intestinal permeability following hemorrhagic shock

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**AIM:** To determine the time course of intestinal permeability changes to proteolytically-derived bowel peptides in experimental hemorrhagic shock.

**METHODS:** We injected fluorescently-conjugated casein protein into the small bowel of anesthetized Wistar rats prior to induction of experimental hemorrhagic shock. These molecules, which fluoresce when proteolytically cleaved, were used as markers for the ability of proteolytically cleaved intestinal products to access the central circulation. Blood was serially sampled to quantify the relative change in concentration of proteolytically-cleaved particles in the systemic circulation. To provide spatial resolution of their location, particles in the mesenteric microvasculature were imaged using **in vivo** intravital fluorescent microscopy. The experiments were then repeated using an alternate measurement technique, fluorescein isothiocyanate...
INTRODUCTION

The small intestinal mucosa normally serves as a selective barrier to uncontrolled transport of large-molecular weight bowel contents into the systemic circulation, while simultaneously allowing the absorption of low-molecular weight nutrients necessary to sustain life. Severe hemorrhagic shock leads to decreased organ perfusion, especially to the bowel. Resultant ischemia to the gut adversely affects its function, and in particular the ability of the small bowel to act as a selective barrier to the uncontrolled egress of luminal molecules into the systemic circulation. It has previously been shown that destruction of the small gut luminal surface occurs very early in shock, and that this destruction appears to be mediated by enzymatic (proteolytic) activity at the bowel mucosa\[^{1,2}\]. Under normal circumstances the bowel is protected from enzymatic degradation by a proteolytically-impermeant mucus layer. Maintenance of this layer requires ATP, and the ability of the protective mucus layer to prevent digestive enzyme attack of the mucosal wall is degraded with ischemia\[^{3}\]. With the mucus layer compromised in shock, digestive enzymes in the bowel are able to destroy the underlying enterocyte layer, cell-cell junctions and the serosa, leading to increases in bowel permeability\[^{4}\].

Enteral infusion of (serine) protease inhibitors into the small bowel lumen has been shown to be protective in multiple forms of experimental circulatory shock that result in gut ischemia, including hemorrhagic, endotoxic, and peritonitis shock\[^{5}\]. Infusion of protease inhibitors enterally (but not systemically\[^{5}\]) prevents or mitigates mortality in different species\[^{5}\] after shock, including man\[^{7}\], presumably by decreasing permeability of the small bowel to inflammatory mediators that otherwise cause systemic inflammation and multiple organ failure\[^{6}\]. However, the mechanisms by which the mitigation of bowel injury improves outcomes after shock are largely unexplored.

Hemorrhagic shock has been reported to increase intestinal permeability, but this has largely been studied ex-vivo (e.g., Ussing chambers\[^{9}\]) or using small markers such as radio-labeled sugars\[^{10}\] or at single or later time points\[^{11,12}\]. As such, the time-course and the extent of bowel permeability changes in this condition are largely unexplored. We hypothesized that proteolytically-derived peptides may be among the earliest mediators to cross the bowel mucosal barrier in shock and sought to determine their time-course in vivo, in order to further delineate the role and timing of bowel-mediated inflammation and remote organ injury in hemorrhagic shock.

MATERIALS AND METHODS

**Ethics statement**

The animal protocol was reviewed and approved by the University of California, San Diego Institutional Animal Care and Use Committee and conforms to the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996).
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85-23, 1996).

**Animals and surgical procedure**

Eight-week-old non-fasted male Wistar rats (300-350 g, Charles River Breeding Laboratories, Wilmington, Mass) were randomly assigned to either hemorrhagic shock \( n = 11 \) or sham shock control groups \( n = 11 \). The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions \( (23 \degree C, 12 \text{ h/12 h light/dark, 50% humidity, ad libitum access to food and water}) \) for over one week prior to experimentation. Animals were not fasted before experiments.

Animals were anesthetized \( (\text{pentobarbital sodium, Abbott Laboratories, North Chicago, IL, } 50 \text{ mg/kg, } \text{im}) \) and the left femoral vein and artery were cannulated to facilitate continuous cardiovascular monitoring and blood withdrawal for the experimental procedure. Further pentobarbital was given intravenously \( (iv) \) as necessary to maintain adequate anesthetic plane. Heparin was given \( (10 \text{ U/mL } iv) \) to facilitate exsanguination and prevent clotting in all animals. The animals breathed spontaneously without tracheotomy.

**Shock protocol**

Hemorrhagic shock was induced by careful removal of blood via the femoral vein in 1 mL aliquots until a mean arterial pressure \( (\text{MAP}) \) of 35 mmHg was achieved. The MAP was maintained at 35 mmHg for 100 min, at which time shed blood was re-warmed to \( 37 \degree C \) and slowly reinfused in 1 mL aliquots, analogous to blood withdrawal, via the femoral vein. Animals were then observed for 100 min \( \text{(Reperfusion)} \) before termination of the experiment \( (\text{Beuthanasia}^\text{®}, 0.22 \text{ mL/kg, } iv) \). This model of hemorrhagic shock has previously been shown to result in ischemia-mediated damage to the small bowel \(^{[2-4]} \). Sham-shock animals were instrumented and manipulated as above without hemorrhage as the comparator group.

**Fluorophore-coupled casein injection into the small bowel**

To determine the ability of bowel-generated proteolytically-cleaved peptides to diffuse into the central circulation, fluorescently-labeled casein \( (\text{EnzChek Protease Assay kit, red-fluorescent BODIPY}^\text{®} \text{ TR-X, Invitrogen, Life Technologies, Grand Island, NY}) \) was injected into the small bowel before the shock procedure \( (n = 6, \text{ both shock and sham shock control groups}) \). The casein is labeled with multiple fluorophores and only fluoresces upon proteolytic cleavage. In confirmatory separate experiments, and because the molecular weight of the fluorescent casein-derived peptides was unknown, fluorescein isothiocyanate \( (\text{FITC}) \)-dextrans 20 \( \text{KDa} \) \( \text{MW} \) was substituted for the fluorescently-labeled casein \( (n = 5, \text{ both shock and sham shock control groups}) \). Before induction of hemorrhagic shock \( \text{or analogous time period in the sham-shock control group}) \) a midline incision was made and the small intestine was carefully exteriorized from the abdomen onto moist warmed \( (37 \degree C) \) gauze. Either one mL casein solution or FITC-dextrans 20 was injected sequentially along the length of the small bowel from the cecum proximally to the duodenum \( (10 \text{ mL of fluorescent solution total}) \).

**In vivo intravital fluorescent imaging of the rat mesentery**

The rat mesentery was gently exposed and draped over a transparent pedestal on a heated animal stage \( (37 \degree C) \) as previously described \(^{[13]} \). The mesentery preparation was continuously superfused \( (2.0 \text{ mL/min}) \) with Krebs-Henseleit solution \( (37 \degree C) \) containing a 95% \( \text{N}_2\)-5% \( \text{CO}_2 \) gas mixture, with care taken to maintain adequate fluid superfusion of the tissue. The mesenteric microcirculation was imaged using an intravital microscope \( \text{[water immersion objective lens } (\times 25, \text{ numerical aperture } = 0.60, \text{ Leitz; Wetzlar, Germany}) \] \) by a color charge-coupled device camera \( (DEI-470, \text{ Optronics Engineering; Goleta, CA; frame rate } 1/125 \text{ s for bright field} \text{ and } 1/2 \text{ s for fluorescence light}) \). All images were recorded \( (\text{Model AG-a270P, Panasonic; Tokyo, Japan}) \) and digitally stored for analysis. Fluorescent images were elicited using a 200-W mercury lamp. The light was passed through a quartz collector, heat filter \( (\text{KG-2, Zeiss; Oberkochen, Germany}) \), and fluorescent filter set \( (\text{Excitation/Emission: } 590/625 \text{ nm for FITC dextran-20 fluorescence (in separate experiments), L3 filter cube, Ploempak, Leitz}) \). Single microscopic fields \( (\text{approximately } 300 \mu \text{m } \times 350 \mu \text{m}) \) containing arterioles and venules were examined. Wright’s stain was used to identify the presence of inflammatory cell types. Briefly, mesentery sectors were excised, fixed in cold acetone \( (10 \text{ min}) \) and subsequently stained with Wright’s stain \( (1 \text{ min}) \). Slides were washed, dehydrated and cover-slipped for imaging.

**Plasma and organ fluorescence assay**

After injection of fluorescent casein into the lumen of the small intestine, blood \( \text{(50 } \mu \text{L}) \) from the femoral artery was collected every 20 min for the duration of the experiment \( (200 \text{ min total}) \) and plasma fluorescence was read immediately \( (\text{SpectraMax Gemini XS, Molecular Devices, Sunnyvale, CA}) \). In animals injected with FITC-dextrans 20, plasma was collected before the shock period and at reperfusion. At the end of the experiments heart, liver and lungs were collected, homogenized and fluorescence readings were performed to determine casein-derived peptide concentrations in these organs.

**Statistical analysis**

Results are presented as mean ± SD, where applicable. Unpaired comparisons of means between two groups in time were carried out using Repeated Measures ANOVA or two-tailed Student’s \( t \)-test where appropriate;
controls (n = 6) (Figure 2). Of note, there appeared to be extensive co-localization of casein-derived peptides with white blood cells in the microcirculation (Figure 3), suggesting that some of these casein-derived peptides may be inflammatory. In order to quantify the increases in bowel permeability to small molecular-weight molecules, in confirmatory experiments the larger FITC-dextrans 20 (molecular weight approximately 20 kD) tracer was injected into the small bowel instead of fluorescent casein at the initiation of the experimental procedures. These results demonstrate a significant increase in measured FITC fluorescence in the plasma of shocked animals (n = 5) compared to sham-shock controls (n = 5) after reperfusion [10.85 ± 6.52 vs 3.38 ± 1.11 fluorescent intensity units (× 10^5, P < 0.05)].

There were also significant increases in permeability-mediated FITC fluorescence after 100 min reperfusion compared to initial values in both shocked animals (10.85 ± 6.52 vs 3.97 ± 4.52, × 10^5, P < 0.05, n = 5) and controls (3.38 ± 1.11 vs 1.44 ± 0.64, × 10^5, P < 0.05, n = 5) (Figure 4). Intravital microscopy of the rat mesentery confirmed increases in microvascular permeability to FITC-dextrans 20 after hemorrhagic shock (Figure 5). However, less extravasation of FITC-dextrans 20 into the surrounding tissues was observed compared to that seen with casein-derived peptides, suggesting a possible differential increase in vascular permeability to the larger FITC-dextrans 20 molecule.

Fluorescence-conjugated casein peptides enter the systemic circulation and circulate in the mesentery tissue and microvasculature
Co-incident with increased plasma concentrations in shock animals, the number of fluorescently-conjugated casein-derived peptides was substantially greater in the parenchyma and microvasculature of the mesentery of shocked animals (n = 6) compared to their non-shocked controls (n = 6) (Figure 2). Of note, there appeared to be extensive co-localization of casein-derived peptides with white blood cells in the microcirculation (Figure 3), suggesting that some of these casein-derived peptides may be inflammatory. In order to quantify the increases in bowel permeability to small molecular-weight molecules, in confirmatory experiments the larger FITC-dextrans 20 (molecular weight approximately 20 kD) tracer was injected into the small bowel instead of fluorescent casein at the initiation of the experimental procedures. These results demonstrate a significant increase in measured FITC fluorescence in the plasma of shocked animals (n = 5) compared to sham-shock controls (n = 5) after reperfusion [10.85 ± 6.52 vs 3.38 ± 1.11 fluorescent intensity units (× 10^5, P < 0.05)].

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Figure 2  Selected *in vivo* microvascular images from two different sham-shock control (A and B) and shock (C and D) animals (*n* = 6, both groups) after hemorrhagic shock or sham-shock and reperfusion. Note the significantly higher levels of red fluorescent casein-derived peptides in the microvasculature and within the interstitium in shock animals (C and D) compared with their sham shock counterparts (A and B).

Figure 3  Representative micrograph with overlays depicting infiltration of white blood cells into the mesentery following shock. Arrows indicate co-localization of fluorescent casein-derived peptide with white blood cells, suggesting a possible inflammatory component to the casein-derived peptides.
Small bowel permeability after mental hemorrhagic shock are gut-derived proteo-
mediators that circulate systemically in early experi-
peptides derived from casein
and/or inflammatory potential is well-established, includ-
organs. That many of these peptides have vasoactive
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bacterial “translocation”, which lessens enthusiasm for
clinical perspective in attempting to modify or mitigate
importantly, there has been very little progress from a
deleterious outcomes in shock
from the bowel to the systemic circulation contributes to
hypothesized that “translocation” of bacterial product
bowel ischemia results in multiorgan failure and shock,
leads to improved outcomes in
experimental shock.[14,15]. The mechanisms by which
bowel ischemia results in multiorgan failure and shock,
are however, incompletely understood. It has long been
hypothesized that “translocation” of bacterial product from
the bowel to the systemic circulation contributes to
deleterious outcomes in shock[16,17], but data supporting
this theory are sparse and contradictory[10,18]. More
importantly, there has been very little progress from a
clinical perspective in attempting to modify or mitigate
bacterial “translocation”, which lessens enthusiasm for
this approach[19].

Alternatively, bowel ischemia, as we demonstrate
here in response to experimental hemorrhagic shock of
non-gastrointestinal origin, leads to very early increases
in microvascular permeability to relatively low molecular
weight products from the lumen of the bowel into the
systemic circulation. Gut-derived peptides lie on a
continuum from approximately 0.1-10 kD, several orders of
magnitude smaller than bacteria and their generated
inflammatory products[20], and can be found in a myriad
number of forms and configurations. Therefore, it is
reasonable to suggest that gut-derived peptides are among
the first molecules from the bowel to enter
the central circulation and subsequently reach remote
organs. That many of these peptides have vasoactive
and/or inflammatory potential is well-established, including
peptides derived from casein[21,22].

We propose that some of the initial inflammatory
mediators that circulate systemically in early experi-
mental hemorrhagic shock are gut-derived proteo-
lytically-generated peptides. Although increased bowel
permeability in response to shock and other stressors has
been well-documented as a general concept[23] and to
fixed molecular weight tracers[24], we demonstrate here
under real-time in vivo observation that in experimental
hemorrhagic shock there is a significant increase in small
bowel permeability compared to sham-shock control
animals within 20 min of ischemia to proteolytically
generated peptide products from the gut, implicating
bowel compromise as an early and perhaps inciting
event in this model. The rapid increase in measured
bowel permeability during early ischemia occurs during
a low-perfusion state with concomitant limited flux of
fluorescein-labelled peptides, implying an under-
estimation of the permeability changes occurring at
the bowel mucosa at this time. Conversely, sustained
increases in small bowel permeability measured after
blood reperfusion, where there is increased flux and
possible “wash-out” of fluorescent tracer, may represent
an over-estimation of increased bowel permeability
rather than a second “reperfusion” injury.

An interesting observation from the study was
the noticeable and frequent co-localization of casein-
derived peptide fragments with possible inflammatory
cells (mast cells vs macrophages, etc.). Although
inflammatory activity of casein-derived peptides has
previously been reported, this has not been directly
confirmed in vivo[25-27]. Further investigation is necessary
to confirm the robustness and clinical relevance of these
findings, as fluorescein-labelled casein and FITC-
dextran 20 were used as markers of permeability and
not for assessment of their intrinsic biologic activities.

DISCUSSION

It has become increasingly apparent that fulminate
circulatory shock, regardless of origin, results in small
bowel ischemia[24]. Likewise, there is increasing evidence
to suggest that prevention of bowel ischemia is be-
 neficial to the organism, and preventing proteolytic
digestion of the bowel mucosa, arising as a consequence
of bowel ischemia, leads to improved outcomes in
experimental shock.[3,14,15].

There are several limitations to this study. Among
these includes our inability to categorize precisely
the molecular weights of the fluorescently-bound
casein-derived peptides secondary to the extreme
heterogeneity of the cleavage products and the non-
linear distribution of the fluorophores on the parent
protein. However, it can be reasonably inferred that
the MW’s of these fluorescent compounds are between
0.1-10 kD (parent compound MW: 19-25 kD) and
smaller than native casein[20].

Previous studies indicate that these peptides are produced in the bowel rather
than proteolytically generated in the circulation after
becoming systemic[3,13,21]. Although there is a lack of
ancillary temporal data correlating in vivo effects with
increases in permeability, confirmatory measurements
using FITC-dextrans 20 support the assertion that in
vivo permeability to larger molecular-weight particles
also increases to some extent in shock[23,24]. Further
studies using calibrated tracers at discrete time-points
and anatomic locations are necessary to completely quan-
tify these findings. Heparin given systemically before
experimental hemorrhage is a possible confounder to our
results when the coagulopathy of hemorrhage/trauma is considered. This is an unavoidable aspect of our hemorrhage model in which blood otherwise clots the catheters and while stored during the ischemic period. An unanticipated result was the heterogeneous accumulation of the fluorescently-labelled peptides in remote tissues. Because of the marked increase in fluorescently-labelled peptides in the mesentery and systemic circulation of animals exposed to experimental hemorrhagic shock compared to sham shock controls it was anticipated that remote tissues would demonstrate similar increases in gut-derived peptide concentrations after shock. The reasons for this lack of difference are unclear but could be due to preferential absorption in other non-measured tissues, heterogeneous accumulation in the selected organs, or simply minimal measured peptide uptake relative to organ tissue mass. Finally, it is acknowledged that the definition of “permeability” as used in these studies is semi-quantitative, in that what is measured is the resulting accumulation of tracer in the measured (vascular or tissue) compartment. As all variables except the changes in permeability are held constant between groups, the permeability results presented here are, strictly speaking, a ratio of permeability changes between the control and shock groups and as such are necessarily semi-quantitative.[32]

In conclusion, this study demonstrates that early increases in small bowel permeability occur during experimental hemorrhagic shock and that proteolytically-derived peptides may be the defining molecules that instigate early inflammation and hemodynamic compromise. Further studies are needed to determine precisely the identity of these putative gut-derived inflammatory mediators and their origin, as well as strategies for preventing their egress into the systemic circulation.

COMMENTS

Background
Ischemia resulting from acute hemorrhage compromises the intestinal barrier, leading to increased permeability of the membrane to bowel-derived contents. Some of these molecules may be intrinsically inflammatory and may possibly contribute to the organ dysfunction and mortality seen in shock.

Research frontiers
The ability of intestinal products, particularly those that are proteolytically generated, to escape into the central circulation following acute blood loss and their subsequent fate is not well understood. The authors were interested in determining in vivo the time course and the extent to which these particles access the central circulation following hemorrhagic shock.

Innovations and breakthroughs
The authors demonstrate in this manuscript that early increases in small bowel permeability occur very early during experimental hemorrhagic shock.

Applications
Proteolytically-derived peptides from the bowel enter the systemic circulation early in shock and may be defining molecules that instigate early inflammation and hemodynamic compromise in shock and associated poor bowel perfusion states.

Peer-review
This is a well written study investigating intestinal permeability after shock in a
rodent model.

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