Regional susceptibility to stress-induced intestinal injury in the mouse


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Novosad VL, Richards JL, Phillips NA, King MA, Clanton TL. Regional susceptibility to stress-induced intestinal injury in the mouse. Am J Physiol Gastrointest Liver Physiol 305: G418–G426, 2013. First published July 18, 2013; doi:10.1152/ajpgi.00166.2013. Injury to the intestinal mucosa is a life-threatening problem in a variety of clinical disorders, including hemorrhagic shock, trauma, burn, pancreatitis, and heat stroke. The susceptibility to injury of different regions of intestine in these disorders is not well understood. We compared histological injury across the small intestine in two in vivo mouse models of injury, hemorrhagic shock and heat stroke, 30% loss of blood volume and heat stroke (peak core temperature 42.4°C). In both injury models, areas near the duodenum showed significantly greater mucosal injury and reductions in villus height. To determine if these effects were dependent on circulating factors, experiments were performed on isolated intestinal segments to test for permeability to 4-kDa FITC-dextran. The segments were exposed to hyperthermia (42°C for 90 min), moderate simulated ischemia (PO2 ~30 Torr, PCO2 ~60 Torr, pH 7.1), severe ischemia (PO2 ~20 Torr, PCO2 ~80 Torr, pH 6.9), or severe hypoxia (PO2 ~0 Torr, PCO2 ~35 Torr) for 90 min, and each group was compared with sham controls. All treatments resulted in marked elevations in permeability within segments near the duodenum. In severe hypoxia or hyperthermia, permeability was also moderately elevated in the jejunum and ileum; in moderate or severe ischemia, permeability was unaffected in these regions. The results demonstrate increased susceptibility of proximal regions of the small intestine to acute stress-induced damage, irrespective of circulating factors. The predominant injury in the duodenum may impact the pattern of acute inflammatory responses arising from breach of the intestinal barrier, and such knowledge may be useful for designing therapeutic strategies.

Loss of the integrity of the intestinal barrier is a well-recognized medical consequence of a wide range of life-threatening stress conditions, such as heat stroke, hemorrhagic shock, trauma, burn injury, pancreatitis, and septic shock. Multiple organ dysfunction syndrome; hemorrhagic shock; heat stroke; ischemia; hyperthermia.

Methods

Mice. Adult male C57BL/6 mice were fed a standardized chow diet and maintained at the University of Florida vivarium on a 12:12-h light-dark cycle until the day of the study. Animals were housed in groups (≥5 mice/cage) at 26°C and 50% humidity. C57BL/6 mice tend to fight for cage dominance, and aggressors were sometimes singly housed when needed. Animals with fighting wounds were allowed to recover fully before entry into the protocol. Several hours before the study, in the early morning, the mice were transported to the laboratory (23–26°C, ~50–70% humidity) and allowed to adapt in their cages for several hours before beginning the protocol. All protocols were approved by the University of Florida Institutional Animal Care and Use Committee. The weights of the animals were not significantly different between groups: 26.5 ± 1.3 (SD) g for control hemorrhagic shock, 27.5 ± 3.1 g for hemorrhagic shock, 27.3 ± 2.2 g for heat stroke control, 25.7 ± 2.5 g for heat stroke, 27.7 ± 2.1 g for in vitro simulated ischemia, and 28.0 ± 3.3 g for in vitro hyperthermia.

Hemorrhagic shock model. The hemorrhagic shock protocol was adapted from Shenkar et al. (53), as modified by Asehnowne et al. (3). Briefly, food and water were provided ad libitum. The animals were anesthetized with 3% isoflurane in O2 within an induction chamber, transferred to a heated surgical surface in the supine position, and maintained under anesthesia with a nose cone. The chest was cleaned with alcohol, and a transthoracic cardiac “stick” was performed using a 29-gauge sterile needle. A calculated 30% of blood volume was withdrawn (0.0275 ml/g mouse, or 0.55 ml/20-g mouse) into a heparinized syringe, which was placed in a sterile test tube in a 37°C water bath. The mice were allowed to recover from anesthesia and blood loss for 60 min. Then they were anesthetized again with isoflurane, and the previously withdrawn blood was reinfused into the retroorbital sinus. Once the blood was injected, the syringe was carefully removed and the mouse was released gently into its cage. The mice were monitored over a 60-min recovery period, anesthetized
The intestinal sacs were filled with oxygenated Medium 199 supplemented with glutamine and tied with 2-0 sutures (46) at a pressure of ∼2–3 cmH2O. Length from the ileocecal junction was measured at each suture site to determine the position of the tissue sample relative to the intact length of the small intestine. After the intestinal sacs were acclimated to 37°C, they were randomly assigned to one of four treatment chambers represented in Table 1 (block randomization to ensure that an equal number of segments were distributed across groups). Each chamber consisted of 7.5 ml of medium and 0.3 mM 4-kDa FITC-dextran (FD4) and exposed to their specific conditions for 90 min.

Analysis of histological samples from in vivo experiments. Transverse sections were cut from the duodenum, jejunum, and ileum for later hematoxylin-eosin staining. To ensure consistency in the samples, mesentery was removed until the intestine could be straightened without straining the intestinal wall. Then 1-cm lengths were cut from (1) the duodenum (1 cm below the pyloric sphincter), (2) the jejunum (half the distance between the pyloric sphincter and the ileocecal junction), and (3) the ileum (2 cm above the ileocecal junction). Histology slides were graded according to the method of Chiu et al. (13) modified to examine individual villi. Two trained raters separately graded each slide. All grading was blind: neither rater knew which samples corresponded to which treatments. All slides for which scores from raters differed by >1 grade were reblinded and regraded. Each slide had multiple cuts of the same sample; raters chose one representative cut based on the fewest staining/cutting artifacts. For the chosen cut, one villus without artifacts was selected, at random, as a starting point. This villus and every 4th villus thereafter were graded and measured until a total of 10 villus measurements were made for that section or, in the case of a large number of artifacts, until no more villi without artifacts were available to measure (a rare event, <3% of samples). Villus height and width were measured using calibrated microscope image analysis. Reductions in villus height were interpreted as indications of ongoing “villi restitution” (10, 14), and crypt depth was used to indicate ongoing enterocyte proliferation, while villus width was used as an indication of injury or swelling.

The results for injury scores were analyzed in two ways. Average scores for a given region and animal were determined, and the grand means were calculated. The resulting populations were not all normally distributed; therefore, the results were analyzed using nonparametric statistics (Kruskal-Wallis ANOVA followed by post hoc Wilcoxon tests; JMP software, SAS). A second frequency analysis of injury scores in all measured villi was performed (∼60 villi per group); then the odds ratios for the presence of injury scores ≥2 in each grouping were determined. Measurements of villus dimensions were parametric and, therefore, analyzed by ANOVA followed by post hoc comparisons.
Data analysis and statistics for isolated intestinal segments. At the end of the experiment, the intestinal sacs were removed from their FD4 baths, their contents were emptied into preweighed tubes, and the surface area of the intestinal sac was measured. Permeability was defined as transport (nmol of FD4/cm² surface area) using the following formula: (concentration of serosal fluid × volume of serosal fluid)/mucosal surface area. This method was described by Lambert et al. (39) for the rat but was adapted for the mouse in a previous study and modified to improve reproducibility in the smaller specimens, where the method was described in detail (46). Changes in permeability as a function of location along the intestinal tract were evaluated using regression software (GraphPad, Prism). Comparison of the residuals in different fitting models resulted in selection of a quadratic formula (2nd-degree polynomial) as the most appropriate fit to describe the regression data. The model was simplified to compare the permeability of all segments measured in the distal half of the intestine with the proximal half. Because these populations were nonparametric, differences were tested using Kruskal-Wallis and nonparametric post hoc analyses (JMP software).

RESULTS

Villus morphology following in vivo hemorrhagic shock and heat stroke. Models of hemorrhagic shock and heat stroke induced marked intestinal injury, which was more predominant in the duodenal regions of the small intestine. Typical histology images of villi from hemorrhagic shock and heat stroke are compared against typical sham controls in both experiments (Fig. 1). When the damage scores for the 10 sample villi from each animal and each region were averaged and grand means were compared (Fig. 2), the injury in the duodenal regions was significantly greater than in matched controls. Similarly, the injury was significantly greater than the measurements in the jejunum or ileum for both models. In contrast, results from samples from the jejunum and ileum were not statistically different from results from their sham counterparts.

To further evaluate the regional nature of the intestinal injury in these disorders, the injury scores of individual villi were analyzed in the frequency domain by plotting frequency histograms. Relative frequencies of each category of injury for ~60 villi in each region are shown in Fig. 3, A and B. For hemorrhagic shock, the odds of a given villus having a damage score ≥2 in the duodenum increased from 2% to 57% and in the jejunum from 20% to 33% compared with sham controls (Fig. 3, A and B). There were no shifts in the distributions for the ileum. Qualitatively similar findings were seen in heat stroke, where the odds of any given villus within the duodenum having a damage score ≥2 increased from 30% in sham controls to 98% in heat stroke. In the jejunum, the odds increased from 12% to 57% and in the ileum from 5% to 23%. Although statistical analyses in the frequency domain are complicated by the multiple samples from each animal, these data provide some insight into the idea that injury was not limited solely to the duodenum but extended in many cases to the jejunum as well.

Average villus height was significantly shortened in the duodenal regions following hemorrhagic shock (Fig. 4 A). In heat stroke, villus height was significantly reduced in the duodenum and jejunum. There were no effects of either stress stimulus on the dimensions of the ileum. Crypt depth and villus width were unaffected by either stress stimulus in any area of the intestine.

Effects of in vitro exposure to hyperthermia and simulated ischemia on regional permeability. Small intestines were isolated from untreated mice, everted, divided into segments distributed along the entire length of the small intestine, and studied for their permeability to FD4 over time. The segments were exposed to one of six different treatment conditions and their associated control conditions. The results for the permeability to FD4 over the 90-min exposure for all groups are shown in Fig. 5. Exposure to moderate and extreme simulated ischemia resulted in marked elevations in permeability to FD4 in the duodenal and jejunal regions and essentially no changes in permeability in the ileum. These data were best fit to a
quadratic equation, and the relationships were highly significant (Fig. 5). In Fig. 5, E and F, differences in permeability between the proximal and distal halves of samples from all experiments are compared. In ischemia, significant differences were only seen in the proximal half of the small intestine. The results were somewhat different in response to severe hypoxia or hyperthermia (Fig. 5, C and D). Although there appeared to be a more prominent increase in permeability in the proximal

Fig. 2. Villus damage grading scores averaged from each animal for each region (n = 5–7 animals in each group). Duo, duodenum; Jej, jejunum. *P < 0.05, **P < 0.01 by nonparametric post hoc analysis following Kruskal-Wallis ANOVA.

Fig. 3. Frequency analysis of the fraction of measured villi in each category of injury (0–5; ~60 villi in each grouping). Odds ratios were determined on the basis of the fraction of villi with damage grades ≥2. In going from control animals, above, to their matched stress animals below, general shifts to the right of the frequency analysis occur.
regions of the intestine, elevations in permeability were also seen in distal areas (Fig. 5, G and H).

**DISCUSSION**

These results demonstrate that, in an acute time frame of 0.5–1 h, the proximal regions of the small intestine in the mouse are more susceptible to gross injury and increases in permeability to high-molecular-weight molecules. Within this acute time frame, this apparent vulnerability does not appear to depend on an intact circulation, circulating inflammatory mediators, invading inflammatory cells, or limitations of blood perfusion to the villus tips. Furthermore, the susceptibility is qualitatively similar in comparatively different models of stress (Fig. 5).

**Comparison with previous work on regional injury and permeability.** Susceptibility of different regions of the small intestine to acute stress-induced injury has only rarely been measured. First, most studies simply omit comparison of the responses of the duodenum with responses of lower regions of the small intestine (10, 21, 43, 45). In the studies available, there is little consistency. With some interventions, predominant injury to the proximal regions of the intestine has been described (33), but damage or apoptosis is also reported to be elevated to a greater extent in the ileum (34), particularly in longer time settings. In many of the previous studies, later time points (6–24 h after the intervention) were observed; in the present study, we observed changes within 30–60 min. The relevance of this is that, at least in response to heat stroke in pigs, significant injury to the duodenum and jejunum occurs within 1 h, whereas injury to the ileum occurs only after 3 h (66). It is well known that damaged villi are restored within 1–3 h (10, 26). Therefore, the relative injury scores or villus dimensions could be markedly different as repair or secondary mechanisms of injury emerge over time. Importantly, later stages of recovery are characterized by recruitment of inflammatory cells, which may induce a different pattern of injury in different regions. For example, neutrophil infiltration following ischemia-reperfusion of the gut is only evident at ~3 h after reperfusion injury (12), far beyond the time frame of this study. Therefore, we believe that our observations are uniquely relevant to early injury, a time that may be most applicable to designing interventions to protect the intestine. Although it is possible that there are species differences, because of the small amount of available data, it is not possible to directly evaluate this possibility, particularly in humans or larger mammals. In vitro studies in rat intestinal segments, no differences in permeability were observed between segmental regions before or after heat exposure (39); in the pig, however, the duodenum and jejunum exhibit predominant injury early in recovery from heat stress (66).

**Potential mechanisms of injury in hyperthermia, ischemia, and hemorrhagic shock.** One common mechanistic thread between the kinds of injury induced by hemorrhagic shock and heat stroke in vivo is the likelihood of local ischemia in both conditions. In the case of hemorrhagic shock, this is presumably due to low cardiac output, low hematocrit, and high levels of circulating catecholamines (42, 55). In heat stroke, ischemia is believed to be due to a combination of the shunting of cardiac output away from the splanchic circulation to allow for peripheral heat exchange and cardiovascular shock in the later stages (29, 30). The PO2 of the villus tip has been estimated to be ~10 Torr less at the tip of the villus compared with the base (6). Since the villi in the duodenum are substantially longer (Fig. 4), it is likely that, for a given PO2 and a reduced blood flow, the degree of hypoxia at the villus tip in the proximal regions would be more pronounced. However, this possibility cannot fully explain the greater susceptibility of the in vitro everted duodenal segments to simulated ischemic conditions. It would be unlikely that a significant PO2 gradient between the base and the apex of the villi would be present, because there was no circulation, and the lumen of the everted intestine is exposed outwardly to a continuously stirred buffer. Therefore, there would be little boundary region or difference in diffusion path, since villi in all regions are of approximately the same thickness (Fig. 4).

Another possibility is that the metabolic rate of the upper intestine, which has been reported to exceed that of the lower intestine (15, 54), would make it more vulnerable to hypoxia. The observation that exposure to severe hypoxia (i.e., near anoxia; Fig. 5C) resulted in essentially all segments of the intestine being affected (Fig. 5C) would favor this possibility.

Fig. 4. Villus height, crypt depth, and villus width in animals exposed to hemorrhagic shock (A) and heat stroke (B), each compared with their respective sham controls. *P < 0.05, **P < 0.01, ***P < 0.001.
In other words, in simulated ischemia with Po2 kept at ~20–30 Torr, there may have been sufficient O2 for the metabolic requirements of the distal regions of the intestine, but the proximal regions, with a higher metabolic rate, may have experienced compromised gas exchange. In severe hypoxia, metabolism in all regions would presumably be O2-dependent, resulting in more uniform damage. Alternatively, our ischemia models included conditions that are not present in hypoxia, including acidosis, hypercarbia, and hyperkalemia (Table 1). One of these factors may have had a protective influence on the distal small intestine that was less effective on the proximal intestine. For example, although intestinal acidosis is generally thought to be damaging to the ischemic ileum (27), in most other tissues or cells that have been studied, acidosis is highly protective during ischemia-reperfusion (7, 64).

Elevated temperature has its own unique impact on the integrity of the intestinal barrier, independent of associated ischemia. This is evident from the effects of hyperthermia alone on the isolated intestinal segments, where Po2 was kept supraphysiological. This isolated effect of temperature has been shown in a number of experimental models: isolated intestinal segments of the rat (39), isolated segments from the mouse (46), and isolated human intestinal cell culture monolayers (23). In isolated mouse intestine, a change in permeability requires exposure to 41.5°C for 30 min (46), which is similar to the requirement of the rat (39). The mechanism is not completely known, but hyperthermia-induced disruption of tight junction organization has been demonstrated in cell culture models exposed for extended periods to 41°C (23). In the isolated segments of the mouse, there does not appear to be a striking difference in vulnerability of the different regions of the intestine to 90 min of hyperthermia, as all regions were substantially affected (Fig. 5C). This more or less similar influence of prolonged heat exposure on permeability did not resemble the pattern of injury observed in the in vivo heat stroke model, where predominant injury was seen...
only in regions near the duodenum. It is possible that permeability
and injury cannot be equated in these in vitro vs. in vivo models
and that the heat stroke model is more closely associated with the
ischemia effects in Fig. 4, A and B. Alternatively, the differences
may simply be due to time and degree of exposure. For example,
the time course of core temperature changes in the in vivo
experiments associated with the injury scores in Fig. 2A was
controlled (46), exceeding 41°C for only ~60 min and exceed-
ing 42°C for ~10 min. This is compared with the exposure of
the isolated segments to 42°C continuously for 90 min. We
speulate that the influence of hyperthermia on permea-
ability across the whole length of the small intestine com-
pared with the more subtle effects in simulated ischemia or
in the in vivo models reflects the “degree” of exposure to heat
or hypoxia, and not a specific relationship between the nature
of the stimulus and the vulnerability of given regions.

Functional significance of duodenal injury to development of
MODS. It is generally held that the predominant negative
consequence of acute injury to the intestinal mucosa is the
translocation of bacteria or bacterial wall fragments into the
bloodstream. This is a primary theory underlying the patho-
genesis of the inflammatory cascades induced by heat stroke
(8, 25) and hemorrhagic shock (51). However, recent studies
have demonstrated that other inflammatory mediators besides
endotoxin cross the intestinal barrier and may be equally, if not
more, important in initiating proinflammatory cascades and
MODS (1, 2, 18, 20, 50). The most promising of these is one
or more naturally occurring pancreatic digestive enzymes (20,
50). Recent studies have demonstrated that blocking protease
activity in the intestinal lumen following several categories of
shock greatly attenuates subsequent mortality and MODS (11,
20). Despite the duodenum’s proximity to the pancreatic duct,
there are substantial levels of pancreatic enzymes throughout
the small intestine. However, the duodenal barrier may be less
equipped than other regions to prevent contact of these en-
zymes with the brush border. For example, rat duodenum has
a lower percentage of mucus-producing goblet cells than other
regions, starting at 4% in the duodenum and gradually increas-
ing to 16% in the distal colon (37). The thickness of the total
rat duodenum mucus layer is ~170 μm, while that of the ileum is 476 μm (4). Also, when this mucus layer is removed by
suction, the ileum replaces mucus at a faster rate than the
duodenum (9). With a decreased thickness and reduced rate of
mucous restoration, the duodenum faces disadvantages to buf-
fer itself against luminal contents. The increased length of the
duodenal villi may contribute to a greater potential for injury.
Finally, longer lengths of villi in the duodenum represent
greater surface areas available for diffusion of pancreatic
enzymes and overall potential for permeability defect.

Another factor is the potential influence of oxidative stress
on this mucus layer and the vulnerability of the duodenal
regions. We previously demonstrated that isolated intestinal
segments exposed to hyperthermia exhibit elevated levels of
oxidative stress and that when the oxidative stress is attenuated
with exposure to the antioxidant N-acetylcysteine, the intestine
is significantly protected from injury and permeability defect in
vitro (46). Interestingly, the mucosal layer itself is highly
sensitive to oxidative stress, and in conditions of hemorrhagic
shock, reactive oxygen-mediated damage is protected by the
free radical scavenger dimethylsulfoxide (24). Therefore, a
reasonable hypothesis is that oxidative stress, induced by
ischemia or hyperthermia, damages the mucosal layer, partic-
ularly in the upper regions of the intestine, making the intes-
tinal wall in this area more susceptible to autodigestion, oxi-
diative stress, or permeability to endotoxins.

The practical significance of these results is that knowledge
of predominant localization of gut injury may help in under-
standing the origins and characteristics of the stress-induced
inflammatory response. Different bacterial wall products influ-
ence specific Toll-like receptors differently throughout the
body. Human cells are equipped with >10 Toll-like receptor
isofoms, and each has a unique sensitivity and physiological
response to specific pathogens (31). These factors may be quite
different when signals arise from breach of the barrier in the
duodenum vs. the ileum or in response to damage-associated
molecular patterns arising from injury in different areas. In
addition, knowledge of the predominant region of intestinal
injury in areas proximal to the stomach may lead to effective
nutritional or pharmaceutical strategies for prevention that
could have rapid access to this region shortly after ingestion.
For example, one recent promising case report used enteral
administration of protease inhibitors to reduce systemic inflam-
mation in a patient with MODS (40), and a similar procedure
was shown to be successful in rodents (11). Such approaches
may be particularly effective when predominant early injury
occurs in the proximal regions of the small intestine. In
addition, it may be possible to provide nutritional supplements
that would have rapid access to the proximal intestine prior to
significant degradation of their activity along the digestive
path. For example, enteral antioxidants (28, 59), metabolic
substrates (38, 48), and polyphenols or flavonoids (44) have
been shown to protect from gut injury following conditions of
ischemia-reperfusion. The mouse could serve as a preclinical
model for development of similar effective strategies.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
V.L.N. and T.L.C. are responsible for conception and design of the research; V.L.N.,
J.L.R., N.A.P., and M.A.K. performed the experiments; V.L.N., J.L.R., N.A.P.,
M.A.K., and T.L.C. analyzed the data; V.L.N. and J.L.R. prepared the figures; V.L.N.,
J.L.R., N.A.P., M.A.K., and T.L.C. interpreted the results of the experiments; V.L.N.
and J.L.R. wrote the draft of the manuscript; V.L.N., J.L.R., N.A.P., M.A.K., and T.L.C.
edited and revised the manuscript; V.L.N., J.L.R., N.A.P., and M.A.K. approved the
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REFERENCES


