Ischemia/Reperfusion Injury Alters Sphingolipid Metabolism in the Gut

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Abstract
Background: Intestinal ischemia/reperfusion injury (I/R) is a significant cause of morbidity and mortality in surgical patients. Ceramide is a mediator of apoptosis and has been implicated as increasing bacterial infection susceptibility. The metabolite of ceramide, sphingosine, was recently shown to play an important role in the cell-autonomous, innate immune response of the upper respiratory tract by killing bacterial pathogens. The role of ceramide and/or sphingosine after mesenteric I/R is unknown. We investigated the specific effects of intestinal I/R on tissue ceramide and sphingosine concentration and resulting susceptibility to bacterial invasion. Methods: To simulate intestinal I/R, C57BL/6 mice underwent 30 minutes of vascular clamp-induced occlusion of the superior mesenteric artery followed by variable reperfusion times. Jejunum segments and intraluminal contents were analyzed for ceramide, sphingosine and bacteria using immunohistochemistry. Jejunum samples were also homogenized and cultured to quantify bacterial presence in the proximal intestine. Results: We hypothesized that I/R induces an increase of ceramide in the intestine resulting in increased permeability, while a concomitant decrease of sphingosine may permit bacterial overgrowth. Control mice had no measurable bacteria in their proximal jejunum as measured by tissue culture and immunohistochemistry. After I/R, bacterial counts in the jejunum increased in a time-dependent manner, reaching a peak at 12 hours after reperfusion. Immunohistochemical analysis revealed a marked increase in ceramide in the vasculature of jejunal villi. In contrast, while ceramide concentrations in the epithelial cells decreased after I/R, sphingosine levels appeared to remain unchanged. Surprisingly, bacteria present in the jejunal lumen following I/R contained a ceramide coat. Conclusion: These data indicate that intestinal I/R leads to small intestine bacterial overgrowth as well as ceramide formation in the jejunal vasculature, which may contribute to the gut permeability associated with this injury. Moreover, our novel finding of ceramide in bacterial membranes represents a new opportunity to investigate the dynamic pathogenicity of the gut microbiome. The hypothesis that a decrease of sphingosine after I/R permits bacterial overgrowth in the intestine was not confirmed.
Introduction

Acute mesenteric ischemia is a common problem with a multitude of causes including systemic hypovolemia, thrombotic or embolic vasoocclusive disease, aortic surgery, small bowel transplantation, and strangulated intestinal hernias [1, 2]. Bowel ischemia, and the subsequent reperfusion injury (I/R), leads to significant local and systemic inflammation that can result in multiple organ dysfunction syndrome and a mortality rate ranging from 30-90% [1]. One proposed mechanism of this inflammation is gut epithelial barrier damage that allows translocation of gut bacteria and toxins to the mesenteric lymph nodes, circulation, and distant organs [2, 3]. Moreover, mesenteric I/R leads to neuromuscular damage and intestinal dysmotility [4-7]. Studies have shown that disruption of gut motility leads to alterations in the gut microflora [8]; however, little is known about the acute impact of mesenteric I/R on bacterial populations in the jejunum.

Ceramide, a sphingolipid generated in part by sphingomyelinase enzymes, has been associated with cell damage and death in a variety of cellular processes [9-14]. Animal models have demonstrated a correlation between intestinal I/R and ceramide metabolism [15-17]. Specifically, increased sphingomyelinase activity and ceramide concentration following I/R have been linked with epithelial apoptosis, histological disruption, lipid oxidation, as well as increased expression of ICAM, Bax, Bcl-3, Fas ligand, and other proinflammatory cytokines [15, 16]. However, the specific source of this increased ceramide and its role in gut pathophysiology remain unclear.

We have recently shown that sphingosine, which is produced by consumption of ceramide by acid, neutral or alkaline ceramidases, is highly expressed in the epithelial cells of the trachea and the bronchi and serves to kill invading bacteria (Tabazavareh, Seitz, Jernigan et al, Cellular Physiology and Biochemistry, in press) [18, 19]. A reduction of sphingosine in tracheal and bronchial epithelial cells was observed in airways of cystic fibrosis mice and patients, correlating with a high infection susceptibility, which was corrected upon inhalation of sphingosine or inhalation of acid ceramidase consuming ceramide to sphingosine. In vitro studies showed that sphingosine kills bacterial pathogens (Tabazavareh, Seitz, Jernigan et al, Cellular Physiology and Biochemistry, in press) [18]. We therefore hypothesized that I/R alters the ratio of ceramide with an increase of ceramide and a decrease of sphingosine in the epithelial and endothelial cells of the jejunum allowing bacterial growth and alterations of the jejunal microflora after I/R and disrupting the tightness of the epithelial and endothelial cell layer.

Materials and Methods

Animal Model

All animal procedures performed were approved by the University of Cincinnati College of Medicine Institutional Animal Care and Use Committee. Male C57BL/6 mice aged 8-10 weeks were purchased from Jackson Laboratories. Intestinal I/R was induced similar to previous reports [20, 21]. In brief, mice were placed under isoflurane anesthesia, their abdomens shaved and prepped with betadine and alcohol, and lower midline laparotomy was performed. The entire jejunum, ileum, and cecum were exteriorized and the mesenteric blood vessels were identified and occluded with a vascular clamp. After an ischemia time of 30 minutes the clamp was removed and visible reperfusion was appreciated. The intestines were replaced and the abdominal wall closed in two layers with silk suture. Mice were sacrificed after a reperfusion time of 1-24 hours and their tissues collected for analysis.

Quantification of Jejunal Bacteria

To characterize the proliferation of gut bacteria into the proximal small intestine, the first 2cm of jejunum (starting at the ligament of Treitz) were removed in a sterile fashion and placed in sterile HEPES/Saline (H/S; 20 mM HEPES, 132 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄, pH 7.4).
These samples were then homogenized with scissors, diluted 1:100 and 1:1,000 in H/S, and plated on TSA plates overnight in a 37 °C incubator. Discrete colony forming units (CFUs) were counted and reported as log values of CFUs per segment of jejunum.

**Immunohistochemistry**

For histological analysis, intestinal segments were fixed in 10% formalin for 48 h, serially dehydrated, and embedded in paraffin for sectioning at 5 µm. Sections were dewaxed and rehydrated, then incubated with pepsin (Digest All, Invitrogen) for 30 min and 37 °C, washed, and blocked for 10 min with PBS supplemented with 5% FCS and 0.05% Tween 20. Next, sections were incubated for 45 min at room temperature with anti-ceramide (GlycoBioTech) or anti-sphingosine (clone NHSPH, Alfresa Pharma Corporation, Japan) antibodies diluted 1:500. The sections were then washed in PBS with 0.05% Tween 20 and incubated again for 45 min at room temperature with Cy3-labeled donkey anti-mouse IgM antibodies (Jackson ImmunoResearch), washed again in PBS with 0.05% Tween 20, washed a final time with PBS, and mounted either in Mowiol or Vectashield with DAPI (Vector Laboratories) for DNA localization.

For a more detailed analysis of intraluminal contents, the proximal jejunum was opened and stool was expressed into 1 mL of sterile H/S, spun at 500 RPM for 5 min to pellet stool debris, and this supernatant was then spun at 4000 RPM for 10 min to pellet bacteria. Bacteria were then resuspended in 4% formaldehyde in PBS and fixed at room temperature for 10 min. Fixed cells were centrifuged at 2800 rpm for 10 min and resuspended in PBS. Fixed cells were diluted 1:100 and 100 µL per sample was placed into the Cytospin3 (Shandon) and spun at 600 rpm for 10 min. Immunocytochemistry was then performed. Samples were blocked with 5% FCS in PBS for 45 min. Next, samples were incubated for 45 min at room temperature with mouse anti-ceramide antibody (GlycoBioTech) diluted 1:500. The sections were then washed in PBS with 0.05% Tween 20, incubated for 45 min at room temperature with Alexa fluor 568-labeled donkey anti-mouse antibodies (Jackson ImmunoResearch) diluted 1:1000, washed in PBS with 0.05% Tween 20, washed a final time with PBS, and mounted with Vectashield with DAPI (Vector Laboratories). Cells and histology sections were imaged using laser scanning confocal microscopy (Nikon A1R GaAsP Inverted Microscope).

**Results**

**Mesenteric I/R Induces Small Intestinal Bacterial Overgrowth**

To study the impact of I/R on the small intestine microbiome and to define the role of sphingolipids in potential alterations of the homeostasis of the intestinal microbiome, mesenteric ischemia was induced for a period of 30 minutes and samples of proximal jejunum were acquired at various time points after reperfusion. As seen in Figure 1, the proximal jejunum had a negligible amount of bacteria in control animals. Mesenteric I/R caused bacterial proliferation into the proximal jejunum, most pronounced at 12 hours, after which bacterial counts appeared to decrease.

**Fig. 1.** Mesenteric I/R leads to small intestine bacterial overgrowth. Mice underwent either no surgery (control) or 30 minutes of mesenteric ischemia followed by 2-24 hours of reperfusion. Mice were sacrificed and a segment of proximal jejunum was cultured aerobically for bacterial counts. Bacterial colonization increased after I/R starting at 4 hours and reaching a peak at 12 hours (n=6 at each time point; *=p<0.05 vs all groups, **=p<0.05 vs control, 2 hours, 24 hours; ANOVA).
Mesenteric I/R Increases Ceramide in the Intestinal Vasculature

It has previously been reported that mesenteric I/R increases ceramide concentrations in the intestine [15-17], however these reports do not specify a location or mechanism. We used immunohistochemistry to further detail the effects of mesenteric I/R on ceramide in the small intestine. Figure 2 shows ceramide content in the jejunum of mice who either received no injury or underwent mesenteric ischemia followed by varying times of reperfusion. Following I/R, ceramide content appeared to increase in the villus vasculature with the most intense staining at 4 hours after I/R. White bar represents 50 μm.

I/R Reduces Ceramide, but Does Not Change Sphingosine, in Intestinal Epithelial Cells

We have previously shown that an increase of ceramide in epithelial cells of the lung increases infection susceptibility of the host to bacterial pathogen [9, 18]. Furthermore, we have demonstrated that sphingosine is expressed at high levels in healthy airways, kills invading pathogens and serves as a first line defense against pathogens in the airways [18]. We therefore hypothesized that I/R induces a change of ceramide and/or sphingosine in intestinal epithelial cells, which then permits bacteria to grow. However, immunostainings for ceramide and sphingosine allowing us to define local changes of the lipids in the intestinal...
epithelial cell layer revealed a decrease of ceramide in the epithelial cell layer after I/R, as early as 2 hours after reperfusion, while sphingosine in the epithelial cells remained stable and unchanged after I/R (Fig. 3). Epithelial sphingosine remained stable even with increasing severity of ischemic injury (45 and 60 minutes, data not shown), indicating that survivable mesenteric I/R does not alter sphingosine expression on the gut epithelium.

**Jejunal Bacteria Demonstrate Increased Ceramide after I/R**

The gut vasculature was not the only area to demonstrate increased ceramide following I/R. While examining tissue sections it was noted that intraluminal bacteria stained positive for ceramide in I/R samples, a feature that was not present in control samples. Figure 4 shows fixed jejunum specimens from control animals and those that underwent I/R. While control mice had little or no ceramide positivity in the lumen, I/R led to a striking increase in ceramide-positive bodies in the lumen.

To further characterize these ceramide-positive bodies, intraluminal contents were isolated from the jejunum of control and I/R mice, fixed, and stained for ceramide along with a DNA counterstain (Fig. 5). Again, luminal contents from control mice had almost no ceramide positivity, and only faintly visible DAPI staining. Samples from I/R mice had marked increase...
in ceramide-positive bodies, which had a morphologic appearance similar to bacterial bacilli and cocci. Specifically, the third panel of Figure 5 shows a bacillus-like structure that is approximately 8 µm in length, with a ceramide-positive membrane and a DAPI-positive interior, indicating the presence of nucleic acids. Taken together, these data suggest that I/R leads to the presence of bacteria in the jejunum that appear to have increased concentrations of ceramide on their membrane.

Discussion

In summary, this manuscript characterizes several consequences of mesenteric I/R on the proximal small intestine. First, we have demonstrated a reproducible murine model of small intestine bacterial overgrowth following intestinal I/R injury. Also, we have identified specific areas of altered ceramide concentrations after I/R: ceramide is increased in the intestinal vasculature and the bacteria that proliferate in the jejunum, but is transiently decreased in the gut epithelium. However, sphingosine expression on the gut epithelium did not change, contrary to our hypothesis. These changes in ceramide metabolism after I/R are novel and interesting as they relate to the pathogenicity of intestinal microbes following an ischemic injury to the intestine. Previous data from our group have shown that ceramide in epithelial cells of cystic fibrosis lungs promotes bacterial infection [9]. Vice versa, healthy airways contain a high concentration of sphingosine, which is largely reduced in cystic fibrosis [18]. We have also shown that sphingosine kills bacteria at rather low concentrations [18]. Further biophysical studies show that sphingosine and ceramide interact in the same membrane domains [22, 23]. It is therefore tempting to speculate, although unproven that the "ceramide coat" of the bacteria might interact with sphingosine and might protect them from the bactericidal effect of sphingosine present in the luminal membranes of the enterocytes.

There is a substantial body of literature on the role of the intestinal microbiome, and several studies have implicated gut bacteria in systemic injury following bowel I/R. Some have shown bacterial counts to increase in the ileum, but not jejunum, following bowel I/R [24, 25]. We have demonstrated that bacterial counts in the jejunum increased after mesenteric I/R, peaking at 12 hours. The return to normal counts presumably occurs as the mice recovered from the injury and the gut resumed its normal levels of peristalsis and acid delivery that regulate the jejunal microbiome [26]. Increased concentrations of microflora in the gut following I/R lead to local tissue damage and tight junction disruption, bacterial translocation to the blood and distal organs, and increased systemic inflammation and cytokine release [27-29]. I/R-induced injury can also be reduced by treatment with antibiotics [30] or with probiotics such as lactobacillus and bifidobacteria that compete with pathogenic bacteria [27, 28]. These findings all suggest that intestinal dysbiosis contributes to local and systemic injury observed following mesenteric I/R, and our murine model of small intestine bacterial overgrowth might serve as a useful tool for studying this phenomenon.

Our investigations also revealed that many, but not all, of the bacteria present in the jejunum after I/R have ceramide on their external membranes. The presence of ceramide on the membrane of bacteria is a previously unreported finding, and thus its role is unknown. Ceramides have been shown to be necessary for fungal growth [31] and virulence [32, 33], and lipid rafts on fungal membranes are known to concentrate virulence factors [34]. Ceramide has also been shown to be involved in the host response to bacterial infections [35, 36]. Indeed, the ceramide on bacteria isolated from the gut appears to cluster on the outer membrane. Perhaps ceramide on the external membrane of gut bacteria plays a role in their pathogenicity. It is also unclear how the ceramide is generated: is it absorbed from the host, generated by bacteria sphingomyelinase, or generated by host sphingomyelinase? All of these possibilities, and a mechanistic understanding may aid in preventing microbe-related injury following intestinal disruption.
Previous studies of mesenteric I/R in rats have demonstrated increased ceramide in the gut after injury via biochemical analysis [15-17]. These studies do not posit a location of ceramide generation, but did correlate increased ceramide with acid sphingomyelinase activity, oxidative damage, and apoptosis. Using fluorescent immunohistochemistry we were able to specifically describe an increase in ceramide in the sub-epithelial region of the jejunal villus, corresponding to the vascular endothelium following I/R. This finding may explain the increased vascular permeability that occurs following mesenteric I/R [28, 37, 38]; animal studies have demonstrated vascular permeability at 4 hours of reperfusion using protein leak studies [15-17]. Perhaps mesenteric I/R leads to endothelial injury, tight junction disruption, and subsequently vascular leak. Future studies may aim to ameliorate this leak by preventing endothelial cell damage and death.

Sphingosine has been shown to play an important role in microbial control, specially in the lungs [19] and on the skin [39]. Sphingosine levels in the upper airways of cystic fibrosis mice and patients are greatly reduced, which leads to increased infection susceptibility; this can be corrected by inhalation of sphingosine or acid ceramidase, which generates sphingosine from ceramide [18]. We hypothesized that mesenteric I/R would cause metabolism of sphingosine to ceramide in the small bowel, which would in turn allow bacterial proliferation. While we did see the bacterial overgrowth following I/R, as well as increased ceramide, we did not see changes in the sphingosine concentrations. However, this does not rule out sphingosine as an important microbial regulator in the gut.

In conclusion, we have demonstrated that mesenteric I/R in mice results in the proliferation of ceramide-positive bacteria in the jejunum as well as increased ceramide in the vasculature of the jejunal villi. The novel finding of a “ceramide coat” on the gut bacteria may suggest a mechanism of pathogenicity following I/R and other intestinal injuries. The gut microbiome is a complicated system that is surely involved in systemic disease, and sphingolipid metabolism likely plays an important role.

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Disclosure Statement

The authors of this study have no disclosures or conflicts of interest to report.

References


