The intestine as source of cytotoxic mediators in shock: free fatty acids and degradation of lipid-binding proteins

Alexander H. Penn and Geert W. Schmid-Schönbein

Department of Bioengineering, The Whitaker Institute of Biomedical Engineering, University of California San Diego, La Jolla, California

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SHOCK AND SUBSEQUENT MULTIPLE organ failure remain the primary causes of late-stage morbidity and mortality in victims of trauma (16, 44). The intestine plays a central role in shock and has been hypothesized to be a primary source of inflammatory mediators (1, 2, 9, 11, 26, 32). Trauma, sepsis, burns, radiation, and other insults may cause ischemia in the gut and increased permeability of the intestinal mucosal layer (5, 37, 45).

Since Litten’s classic experiment in which he ligated the superior mesenteric artery in dogs, it has been known that a key feature of shock is intestinal necrosis (25). Necrosis of the intestine contributes to fluid loss on reperfusion, lack of nutrient absorption, and inflammatory mediator release (1, 10) and may contribute to inflammation in shock. It is, therefore, important to understand mechanisms associated with intestinal necrosis.

The increased permeability of the intestinal mucosal layer mentioned above has been linked to inflammation and to necrosis of the gastrointestinal tract (1, 9, 12, 27, 40). When mucosal permeability is enhanced during shock, i.e., the barrier provided by the mucin layer and other defenses fails, digestive proteases enter the wall of the intestine and initiate self-digestion of the intestinal wall (36). Inhibition of enzyme activity in the lumen of the intestine during intestinal ischemia serves to reduce the presence of inflammatory mediators in the plasma and improve symptoms of shock (30, 31).

Ischemia may not be the only direct cause of necrosis within the intestine. We showed recently that homogenates of ischemic intestines, but not nonischemic intestines, contain cytotoxic mediators that cause necrosis in naive cells (34). This cytotoxicity is due to pancreatic digestive enzymes in the intestinal lumen acting either on the tissue in the wall of the intestine or on ingested food (34). We established a model for generation of cytotoxic factors by digesting homogenates of intestinal wall tissue with selected purified pancreatic proteases or with a mixture of digestive enzymes from the fluid in the lumen of the intestine.

Utilizing this model, we determine here the nature of the cytotoxic factors and their mechanism of formation. We will show that the cytotoxicity is due to formation of free fatty acids (FFAs) by an enzyme with lipase activity in the intestinal wall. Cells exposed to the intestinal homogenates are protected from death by proteins, like albumin, that bind the FFAs in the homogenate, unless those FFA binding proteins are themselves digested by proteases.

MATERIALS AND METHODS

Materials. 1-Chloro-3-tosylamido-7-amino-2-heptanone (TLCK)-treated chymotrypsin and pancreatic elastase were obtained from Worthington Biochemicals (Lakewood, NJ), and chloroform, methanol, dimethyl sulfoxide, dimethyl formamide, ethanol, porcine pancreatic lipase, bovine serum albumin (BSA), rat serum albumin (RSA), the lipase inhibitor tetrahydrodiprotinin (orlistat), NaCl, NaH2PO4, NaN3, polyethylene glycol, dextran 229, Histopaque-1077, Percoll, and propidium iodide (PI) were obtained from Sigma Chemical (St. Louis, MO). Bovine pancreatic trypsin inhibitor (aprotinin) was obtained from Bayer Pharmaceuticals (West Haven, CT), hydrophobic borosilicate glass-fiber filters (Pall Gelman, East Hills, NY) from Fisher Scientific (Pittsburgh, PA), and oleic acid and NEFA C kit for determining FFA concentrations were obtained from Wako Chemicals (Richmond, VA) and NEFA C kit for determining FFA concentrations was obtained from Wako Chemicals (Richmond, VA).

Address for reprint requests and other correspondence: A. H. Penn, Dept. of Bioengineering, The Whitaker Institute for Biomedical Engineering, Univ. of California, San Diego, La Jolla, CA 92093-0412 USA (e-mail: apenn@bioeng.ucsd.edu).

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(Nuess, Germany). O-pivaloyloxymethyl umbelliferone (C-POM) and the Enzcheck protease activity kit (E6639) were from Invitrogen (Carlsbad, CA). Rat albumin anti-serum was purchased from Immunology Consultants Laboratory (Newberg, OR). Coomassie blue G-250 Protein Assay Reagent was obtained from Pierce Biotechnology (Rockford, IL). Aprotinin was a generous gift from Dr. Erik Kistler.

**Surgical procedure and organ collection.** All animal protocols were reviewed and approved by the University of California San Diego Animal Subjects committee. Male Wistar rats were given general anesthesia (pentobarbital sodium, 50 mg/kg im) and cannulated via the left femoral vein. The animals were then euthanized (120 mg/kg iv pentobarbital sodium). The entire small intestine distal to the pancreatic duct was harvested and cut into three to four sections to simplify later removal of luminal contents (solid and semiliquid content of the small intestine containing partially digested food and digestive enzymes, etc.) and rinsed in saline. The entire luminal contents with digestive enzymes and food contents were removed by manual peristaltic compression. The sections of intestine were slit open longitudinally and placed together in a sealed centrifuge tube with 40 ml of saline and agitated to remove residual luminal contents and/or digestive enzymes from the mucosal surface and then transferred to another tube with 40 ml of saline and agitated. The rinsed intestine was placed into tubes, weighed, and frozen (−80°C) until homogenization. For selected experiments, the luminal contents were also saved, weighed, and frozen for later homogenization. Rapid freezing at every step was found to be important to minimize the action of any digestive enzymes in the intestine or luminal content before the experiments.

**Tissue homogenization.** Six milliliters of cold phosphate-buffered saline (PBS) per gram of tissue were added to frozen intestine or luminal content. The intestines and luminal content were then homogenized in a 9:1 ratio (vol/vol) with PBS or 50 mg/ml RSA. Samples were then tested for cytotoxicity.

To examine lipase inhibition, organ collection and preparation were carried out as described above with the following modifications. For the collection of small intestine, small adjacent segments of rinsed intestine (−0.1 g) from the midpoint between the start of the jejunum and the terminus of the ileum were dissected and separately weighed and frozen for later homogenization. The pieces of intestine were homogenized in 6 ml/g tissue of lipase inhibitor (0.25 mg/ml orlistat, <1.25% ethanol final) or PBS (with an equivalent amount of ethanol as a control). Centrifugation was as above, but the samples were not filtered at the end due to the small volume. The supernatant was aliquoted and frozen for later testing of lipase activity.

Some aliquots were mixed 5:1 with 3 mg/ml pancreatic elastase (0.5 mg/ml final) and incubated for 6 h at 37°C, then frozen again for later testing of protease activity and cytotoxicity. Elastase was used here as opposed to chymotrypsin, as preliminary studies revealed that orlistat also acts as a chymotrypsin inhibitor (inhibited ~25% of the chymotrypsin activity) but not an elastase inhibitor.

**Ex vivo prevention of cytotoxic mediator formation.** To test our findings under conditions closer to those of shock in vivo, we harvested from each of five male Wistar rats five 2-in. segments of intestine from adjacent positions at approximately the midpoint between the start of the jejunum and the terminus of the ileum. After sealing one end of each segment to leave the luminal content in place, we added 0.5 ml of saline, 0.25 mg/ml orlistat, aprotonin (10,000 Kallikrein-inhibiting units (KIU)/ml), or 0.25 mg/ml orlistat + aprotonin (10,000 KIU/ml) to the lumen of the segment and sealed the other end. One segment was sealed without adding any fluid to the lumen. The segments were then submerged in saline and incubated at 37°C for 3 h. The segments were then removed from the saline, functionally produced more sediment than the digested wall homogenates. Using glass pipettes, we transferred the aqueous fractions into separate glass tubes in which they were lyophilized. We then transferred the lipid fraction into glass tubes and dried them in a nitrogen blow-down apparatus. The remaining sediment fractions were lyophilized in their original glass tubes.

We reconstructed the aqueous fraction back to its original concentration in 6 ml of deionized water (as the salts in the original solution stayed in the aqueous-soluble fraction). The sediment was reconstructed in 6 ml of PBS, and the lipid was reconstructed in 30 μl dimethyl sulfoxide followed by 6 ml of PBS. Aliquots of the solutions were then tested for neutrophil cytotoxicity. The rest was frozen.

In selected recombination studies, the solutions were thawed, and the lipid fraction was mixed with equal volumes of PBS, aqueous fraction, or sediment fraction and then tested for their cytotoxic activity.

**Albumin protection studies.** To determine whether intact albumin or albumin digested by proteases can reduce the cytotoxic activity of the intestinal homogenates, we mixed digested wall homogenate (homogenate mixed with equal volume of luminal fluid, 6 h, 37°C) in a 9:1 ratio (vol/vol) with PBS, 50 mg/ml BSA, or digested BSA (50 mg/ml BSA, 1 mg/ml chymotrypsin, and 1 mg/ml elastase, 6 h, 37°C). Samples were then tested for cytotoxicity.

BSA (50 mg/ml) or PBS was also mixed 1:9 (vol/vol) with the lipid fractions of intestine, intestine + chymotrypsin, and intestine + luminal fluid mentioned above and tested for cytotoxicity.

Finally, to determine whether RSA was as effective as BSA at reducing cytotoxic activity, we mixed digested wall homogenate (homogenate mixed with equal volume of luminal fluid, 6 h, 37°C) in a 9:1 ratio (vol/vol) with PBS or 50 mg/ml RSA. Samples were then tested for cytotoxicity.

**Cytotoxic mechanisms.** To look for mechanisms resulting in cytotoxicity, we digested wall homogenate with an equal volume of 1 mg/ml chymotrypsin, luminal fluid, or PBS (6 h, 37°C) and then froze aliquots of the homogenate for later testing of cytotoxicity, protease activity, lipase activity, FAA concentration, and rat albumin concentration.

**Lipase inhibition.** To examine lipase inhibition, organ collection and preparation were carried out as described above with the following modifications. For the collection of small intestine, small adjacent segments of rinsed intestine (−0.1 g) from the midpoint between the start of the jejunum and the terminus of the ileum were dissected and separately weighed and frozen for later homogenization. The pieces of intestine were homogenized in 6 ml/g tissue of lipase inhibitor (0.25 mg/ml orlistat, <1.25% ethanol final) or PBS (with an equivalent amount of ethanol as a control). Centrifugation was as above, but the samples were not filtered at the end due to the small volume. The supernatant was aliquoted and frozen for later testing of lipase activity.

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Weighed, and frozen for later homogenization and testing on neutrophils.

**Neutrophil isolation.** Fresh human neutrophils were isolated with Percoll gradients from heparinized whole blood obtained from healthy volunteers in the laboratory and resuspended in PBS at room temperature to a concentration of 2 × 10^6 neutrophils/ml (33). This procedure reproducibly resulted in >95% neutrophil purity, as confirmed by nuclear staining with crystal violet in 3% acetic acid.

**Neutrophil morphology.** To determine cell morphology, we mixed 100 μl of isolated human neutrophils with 100 μl of PBS or 100 μl of lipid, aqueous, or sediment fraction of wall homogenate digested with luminal fluid for 6 h at 37°C. Cells were fixed in 1% glutaraldehyde after 20 min and viewed using light microscopy after staining with an equal volume of crystal violet.

**Flow cytometric analysis of neutrophil cytotoxicity.** To measure cytotoxicity with flow cytometry, 100 μl of sample were mixed with 100 μl of neutrophils (10^6 cells/ml final). At selected instances of time, 200 μl of 2 μM PI, a life or death indicator, were added. Within seconds after addition of PI, each sample was tested in the flow cytometer (Becton-Dickinson FACScan, Franklin Lakes, NJ).

The flow cytometric analysis of the neutrophils was carried out, as previously described (34), in the form of percentage of cells that are PI positive, i.e., “dead”.

**Protease activity.** The proteolytic activity was determined using a serine protease activity kit. The substrate used for measuring protease activity was casein, derivatized with pH-insensitive fluorophores. Fluorescence was measured in triplicate by a spectrophotometer (SpectraMax Gemini XS) using the Softmax Pro software (Molecular Devices, Sunnyvale, CA) and expressed as relative fluorescent units (RFUs).

In each well, 16 μl of sample and 64 μl of digestion buffer were mixed with 80 μl of the protease substrate solution at 37°C. Measurements of fluorescence were made every minute for 60 min. Pilot studies showed that the fluorescence usually approached its maximum within that time. Therefore, we reported fluorescence values at the 60-min time point.

**Lipase activity.** To measure lipase activity, we used the fluorescently labeled C-POM lipase substrate (23). In brief, we added 181 μl of 50% dimethylformamide (aq) to 100 μg of C-POM to create a 2 mM stock solution that could be stored at 4°C. We diluted the stock of 50% dimethylformamide (aq) to 100 μl of C-POM working solution. In a 96-well plate, we combined a 20-μl sample with 40 μl of working solution per well. All samples were measured in triplicate (except for the initial study shown in Fig. 3 in duplicate). Measurements were taken in a spectrophotometer every minute for 1 h at 37°C (excitation/emission wavelength = 360/460 nm).

**FFA concentration.** Nonesterified or “free” fatty acid concentrations were determined using an enzymatic test kit (NEFA C, Wako Chemicals, Nuess, Germany). In a 96-well plate, to 10 μl of sample, we added 50 μl of Reagent A/well, and, after 15 min at 25°C, 100 μl of Reagent B/well. After 15 min at 25°C, we measured optical density at 560 nm. Measurements were performed in triplicate. Oleic acid standards served to calibrate the measurements.

**Rat albumin concentrations.** Albumin concentrations were determined by an immunoturbidimetric method adapted from Kleine and Merten (22). Twenty microliters of sample were mixed with 260 μl of buffer solution (0.15 M NaCl, 0.05 M Na₂HPO₄, 1 mM Na₂EDTA, 50 g/l polyethylene glycol) and 20 μl of RSA-anti-serum (diluted 1:1 with saline). We measured absorbance at 366 nm in a quartz cuvette.

**In vivo shock studies.** Male Wistar rats tranquilized with xylazine (2 mg/kg im) and then given general anesthesia (pentobarbital sodium, 50 mg/kg im) were canulated via the left femoral vein for additional anesthetic administration and the left femoral artery for blood collection and pressure measurement. Arterial blood (400 μl) was collected in heparin at this time and centrifuged (1,000 g, 10 min) to obtain plasma, which was then frozen (−20°C). We then performed a laparotomy and exteriorized the small intestine and cecum, which were sandwiched between two pieces of gauze soaked in warm saline, rested against the side of the animal, and covered in plastic wrap to keep in heat and moisture. The superior mesenteric and celiac arteries were then isolated with umbilical tape. Warm saline, 0.25 mg/ml orlistat, aprotinin (10,000 KIU/ml), or 0.25 mg/ml orlistat + aprotinin (10,000 KIU/ml) were then luminally injected into two or three sites along the jejunum and ileum (i.e., distal to the pancreatic duct). Volumes varied from 6 to 8 ml and were adjusted to provide fluid along the entire length of the small intestine without stretching the intestinal wall (we have seen previously that overstretching of the small intestine and especially the duodenum can cause drastic drops in blood pressure). Splanchnic arterial occlusion (SAO) was initiated by tying the umbilical tape isolating the superior mesenteric and celiac arteries around short pieces of hard tubing placed adjacent to the arteries. Note that, because of its close proximity to the superior mesenteric artery and our concern that it should remain undamaged, the main lymphatic vessel exiting the intestine was isolated and occluded together with the superior mesenteric artery. We observed an increase in the mean arterial blood pressure of all animals undergoing intestinal ischemia in this fashion. The wet gauze and plastic wrap were adjusted to cover the intestine and the abdominal opening, leaving the small intestine outside the body for the remainder of the ischemia and reperfusion to allow for easy observation of hemorrhage within the intestine. After 2 h of ischemia, reperfusion was initiated by sliding the tubing out of the occlusion site and then cutting through and removing the umbilical tape. A drop in mean arterial blood pressure always accompanied reperfusion. Arterial blood was sampled at 30 min postreperfusion and treated as above to obtain plasma (frozen at −20°C). Animals were then observed for up to 3 h for survival. Survivors were euthanized (120 mg/kg iv pentobarbital sodium). Plasma was later tested for total FFA and protein concentration.

For some studies, no fluid was injected into the lumen of the intestine, and, in the ischemic group, occlusion duration was 90 min instead of 120 min. Animals were euthanized after the second blood collection at 30 min postreperfusion, and the ileum and jejunum were collected (with luminal content remaining in place), weighed, and frozen (−20°C) for later homogenization (as above). In the nonischemic group, the intestine was exteriorized as before, but the splanchnic arteries were neither isolated nor occluded. Second blood collection, followed by euthanization and intestinal collection, was performed 120 min after the small intestine was exteriorized.

**Protein concentration.** To assay for protein concentration, 10 μl of plasma already diluted 50-fold with saline were mixed with 500 μl of Coomassie blue reagent, and then absorbance was measured in triplicate at 590 nm.

**Statistics.** Unless indicated otherwise, measurements are summarized as means ± SD. When the total number of pairwise comparisons being performed was less than six, differences between multiple groups were tested using theBonferroni correction to the two-tailed paired Student’s t-test. P values were considered significant for P < 0.05 per total number of comparisons being made. For total number of comparisons greater than or equal to six, we use the Tukey-Kramer honestly significant difference test for significance using an α of 0.05. This test does not produce a “P value”.

**RESULTS**

**Lipid fractions but not protein fractions are cytotoxic.** As shown previously (34), unfraccionated intestinal wall homogenate was not cytotoxic (Fig. 1A, first bar), while unfraccionated wall homogenate digested with chymotrypsin or luminal fluid was cytotoxic (Fig. 1, B and C, first bar). However, the aqueous fraction and proteinaceous sediment fractions of digested and undigested homogenates alike were not significantly cytotoxic. Instead, we found that the lipid fractions from
both digested and undigested wall homogenates were the most cytotoxic of all samples tested (Fig. 1, A–C, second bar).

To determine whether the protein fractions have the ability to reduce cytotoxicity in the unfractionated, undigested homogenates, we recombined the lipid fractions with PBS or with the aqueous or sediment fractions. We found that the protein containing aqueous and sediment fractions dramatically reduced the cytotoxicity of the lipid fractions (Fig. 1A, right side). If those aqueous protein fractions or the proteinaceous sediment fractions were digested by protease, they did not significantly protect against lipid toxicity (Fig. 1, B and C, right side).

Since luminal fluid with its mixture of pancreatic enzymes has more protease activity than 1 mg/ml chymotrypsin alone (see Fig. 6B below), it is not surprising that the protein fractions digested by luminal fluid had no protective ability,

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Fig. 1. Cell death after 30-min exposure to separated fractions or unseparated solutions of intestinal homogenate digested for 6 h at 37°C with PBS (as control) (A), chymotrypsin (B), or luminal fluid (C). Fractions consisted of the reconstituted lipid and aqueous phases, as well as the reconstituted sediment that did not enter either phase. Additionally, lipid fractions were mixed 1:1 with PBS, aqueous, or sediment fractions. Values are means ± SD; N = 3 animals. *Forms a significantly different pair; **P < 0.05 vs. unseparated intestinal control; ***P < 0.05 vs. unseparated intestinal control and unseparated intestine + chymotrypsin [Tukey-Kramer honestly significant difference (HSD) test].
while the chymotrypsin digested protein fractions still retain some ability to protect cells from death (Fig. 1, B and C, final three bars).

**Neutrophil morphology after exposure to cytotoxic lipids.** The majority of neutrophils in PBS have a round shape or, in the case of activated cells, are extending pseudopods (Fig. 2, *top row*). Cells exposed to the protein sediment fraction of wall homogenate after digestion with luminal fluid were also round and showed no overt signs of cell membrane damage (Fig. 2, *third row*). In contrast, cells exposed to the lipid fraction were entirely destroyed and barely recognizable as former neutrophils (Fig. 2, *second row*). Cells exposed to the aqueous fraction showed evidence for membrane blebbing (Fig. 2, *fourth row*), similar to what we saw with unfractionated di-

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**Fig. 2.** Four representative neutrophils fixed and stained with crystal violet after 20 min exposure to PBS or the lipid and sediment fractions of intestinal homogenate digested with luminal fluid (first 3 rows), or the aqueous fraction with or without BSA (last 2 rows). P, pseudopod; B, bleb. Bars = 10 μm.
gested homogenates (34), indicating that, although the cells were not yet dying (as seen in Fig. 1), they were damaged. It is possible that this low level of cell damage could be due to incomplete separation of lipids into the lipid phase, as shown by measurement of 98 ± 0.08 μM FFAs in the aqueous fraction and by the prevention of bleb formation with addition of BSA (Fig. 2, bottom row) (see below). The aqueous fraction of undigested wall homogenate caused no detectable bleb formation (not shown).

Lipase activity in the intestinal wall homogenate. One type of lipid that is known to be cytotoxic in high concentration is the nonesterified fatty acid or FFA. FFAs in high concentration form a detergent and, therefore, cannot be maintained in the body in an unbound free form. Instead, FFAs are incorporated into triglycerides or phospholipids or bound to FFA carriers, like albumin (14).

To determine whether FFAs may be present in the wall homogenates, we first measured the lipase activity in the solutions. Lipases release FFAs from triglycerides. The lipase activity in wall homogenate was high enough to digest the available substrate in the assay in minutes (Fig. 3). The activity was significantly higher than measured with purified pancreatic lipase at 5 mg/ml. Wall homogenates that had been digested with chymotrypsin or luminal fluid, or luminal fluid on its own, had significantly lower levels of lipase activity.

Albumin protects against cytotoxic lipids. To test the hypothesis that a FFA binding protein, such as albumin (14, 18, 38), may protect against the cytotoxic FFAs in our homogenates, we added BSA [at 5 mg/ml, a concentration slightly lower than found in the interstitial space in humans (8)], digested albumin, or PBS to digested wall homogenate. We found that albumin was protective but that digested albumin was not (Fig. 4A). BSA at the same concentration blocked the cytotoxicity generated by the lipid fractions of both digested and undigested homogenates (Fig. 4C). RSA was as effective as BSA at preventing cell death (Fig. 4B).

Cytotoxicity is determined by both FFA and albumin concentrations. To directly determine the levels of FFA and native albumin and determine whether those values correlate with the levels of cytotoxicity, lipase, or protease activity, we digested wall homogenates with chymotrypsin, luminal fluid, or with PBS as a control. As reference, we also determined the cytotoxicity of various concentrations of oleic acid in PBS.

Cell death is significantly elevated vs. control at oleic acid concentrations on the order of 10 μM, with complete cell death at 100 μM oleic acid (Fig. 5). The levels of FFAs in all of our

Fig. 3. Fluorescence vs. time due to digestion of a fluorescent lipase substrate by intestinal homogenates digested with chymotrypsin, luminal fluid, or PBS as control or by luminal fluid, chymotrypsin, 5 mg/ml porcine pancreatic lipase, or PBS as controls. Values are means ± SE; N = 3 animals for groups with intestinal homogenate or luminal fluid. *P < 0.05 vs. intestine + luminal fluid, intestine + chymotrypsin, or luminal fluid (paired Student’s t-test).

Fig. 5. Neutrophil death after 1-h exposure to oleic acid. Values are means ± SD; N = 3. *P < 0.05 vs. 0 μM; **P < 0.05 vs. 0, 1, and 10 μM (Tukey-Kramer HSD test).
intestinal wall homogenates were in excess of 300 μM equivalents of oleic acid, more than enough to cause total cell death if unbound (Fig. 6A). The levels of FFA in chymotrypsin digests were lower than those in luminal fluid-digested or undigested wall homogenates (Fig. 6A). We also homogenized small segments of small intestinal wall from five rats in the presence of 4 mM PMSF (this concentration is not biocompatible, but was determined in pilot studies to be capable of inhibiting ~95% of the lipase activity; not shown). We measured FFA concentrations in those homogenates to be 0.37 ± 0.12 mM oleic acid equivalents (compared with 0.44 ± 0.19 mM in the undigested wall homogenates shown in Fig. 6A), suggesting FFAs are present even without additional lipase digestion after homogenization.

Compared with the control wall homogenate (intestine), average protease activity was significantly increased in wall

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**Fig. 6.** Intestinal wall homogenate mixed with PBS, chymotrypsin, or luminal fluid and incubated at 37°C for 6 h and then tested for free fatty acid concentration (A), protease activity after 1 h (B), lipase activity after 20 min (C), cell death after 1 h (D), and rat albumin concentration (E). Values are means ± SD; N = 5 animals. *P < 0.017 vs. intestine or intestine + luminal fluid (A). *P < 0.00004 vs. intestine; #P < 0.00008 vs. intestine + chymotrypsin (B). *P < 0.02 vs. intestine; #P < 0.003 vs. intestine + chymotrypsin (C). *P < 0.008 vs. intestine (note: P = 0.057 for intestine + chymotrypsin vs. intestine) (D). *P < 0.004 vs. intestine (note: P = 0.056 for intestine + chymotrypsin vs. intestine + luminal fluid) (E) (paired t-test). Note for A–D, the values for the nonintestine-containing controls are shown for reference, but, as there were no animals to “pair” the results, they are not included in the statistical analysis.
homogenates digested with chymotrypsin or luminal fluid (intestine + chymotrypsin or intestine + luminal fluid) (Fig. 6B), by an amount approximately equal to the protease activity of chymotrypsin or protease activity in the luminal fluid alone, respectively. The protease activity in luminal fluid digests of wall homogenate was also significantly greater than the protease activity of the chymotrypsin digests.

Similar to the results in Fig. 3, we observed a high level of lipase activity in the undigested wall homogenate (Fig. 6C). This dropped significantly after incubation with chymotrypsin or luminal fluid. To determine whether the luminal fluid may have higher lipase activity before autodigestion by proteases during incubation, we tested the lipase activity of the luminal fluid used for the digestions in Fig. 6, with or without incubation. Without incubation, the fluorescence levels (at 20 min) were 989 ± 84 RFUs compared with 689 ± 6 RFUs with incubation. Although greater before incubation, the lipase activity of luminal fluid was small compared with the values we encountered with intestinal homogenate (Fig. 6C).

Cell death increased significantly after digestion with luminal fluid but, after chymotrypsin digestion, was only marginally (not significant) elevated (Fig. 6D). Rat albumin levels decreased significantly after protease digestion by either chymotrypsin or the mixed pancreatic digestive enzymes in the luminal fluid (Fig. 6E).

To examine the connection between FFA formation and albumin digestion and cell death, we correlated cell death generated by each individual undigested or digested homogenate with FFA and with albumin levels (Fig. 7). This particular analysis shows that cell death only occurs when there is both a high level of FFA and a low level of albumin. Moreover, chymotrypsin and luminal fluid reduced the immunodetected albumin levels of each animal’s homogenate to approximately the same level. However, looking at the values in animals 2 and 5 and to a lesser extent animal 1 (the animals for which one or both digested homogenates caused <100% cell death), we see that, for a given animal, lower levels of FFA (usually in the chymotrypsin digest) produce less cell death.

Lipase inhibition prevents cytotoxicity. To determine whether cytotoxicity in protease digests of wall homogenate is the result of FFA acid formation and release, as opposed to the formation of a cytotoxic factor directly by proteases, we inhibited lipases in homogenates to prevent FFA formation without interfering with protease activity during subsequent protease digestion. In a pilot study, we determined that the lipase inhibitor orlistat also inhibits chymotrypsin. Therefore, for this study, we instead digested the wall homogenates with pancreatic elastase. We add the orlistat into the homogenization buffer, since the pilot studies demonstrated that the lipolytic enzymes in the intestine require only short times to produce cytotoxic lipids.

Orlistat, while not able to completely prevent lipase activity, was able to significantly reduce its activity (Fig. 8B). Its blockade was sufficient to significantly reduce cell death in the elastase digest (Fig. 8A) without affecting the protease activity (Fig. 8C).

An ex vivo shock study. To explore the ability of orlistat and/or the protease inhibitor aprotinin administered to the lumen of the intestine to limit cytotoxicity in a model of intestinal ischemia in shock, we cut the intestine into 2-in. lengths. Each segment of intestine was sealed on each end (after addition of saline, orlistat, aprotinin, or orlistat plus aprotinin into their lumen) and incubated in saline at 37°C for 3 h to simulate total ischemia before homogenization. We found that, on average, both the controls without added fluid and the saline groups caused cell death, although the degree of cytotoxicity varied considerably from animal to animal and even between the two groups in the same animals (Fig. 9).

Aprotinin-treated segments also had a high level of variability (one caused <1% cell death, while another caused >98% cell death). Orlistat treatment alone significantly reduced cell death compared with the control group with no fluid added. It prevented cell death in four of six cases and reduced its severity in the other two cases.

Orlistat plus aprotinin, however, was able to eliminate cell death in all cases and had significantly reduced cell death compared with both the control group with no fluid added and the aprotinin group.

Ischemic vs. nonischemic intestine. To test the likelihood that the cell death we had previously seen with ischemic, but not nonischemic, intestinal homogenates is due to the same mechanism of FFAs as in protease-digested homogenates of intestinal wall, we homogenized the entire jejunum and ileum of rats subjected to intestinal ischemia via SAO for 90 min followed by 30 min of reperfusion. Sham rats were kept nonischemic. To these homogenates, we added BSA or PBS and measured cell death of cells exposed to these mixtures. As seen previously (34), the homogenates of ischemic intestine were highly cytotoxic (Fig. 10). The nonischemic intestine’s homogenate was significantly less cytotoxic, although its cytotoxicity was not negligible (of the four intestines in that group, only the one harvested closest to the day of homogenization showed no signs of causing cell death, suggesting that −20°C may not be sufficiently cold to prevent some protease degradation in intact frozen intestines over time). However, cell death by either ischemic or nonischemic intestinal homogenates was completely prevented by the addition of BSA (the
reduction in the nonischemic case from 31.1 ± 20.7 to 0.5 ± 0.3 with BSA was nearly significant with $P < 0.06$.

To begin to probe the question of whether the intestinal lipases we observed in homogenates are located in a compartment available to proteases entering the intestinal wall during shock, we measured lipase activity in the homogenates of ischemic and nonischemic intestines. We observed that the mean lipase activity of the ischemic intestine was reduced, but not significantly, compared with the mean lipase activity of the nonischemic intestines (Fig. 11). In contrast, the digested intestinal wall homogenate had significantly less lipase activity than the undigested homogenate, suggesting protease digestion of lipases.

To determine whether the FFA concentration is increased in the circulation following reperfusion in the SAO model of shock, we measured total FFA concentration in the plasma of rats before 90 min of ischemia and after 30 min of reperfusion

Fig. 8. Cell death after 60-min incubation with neutrophils (A), the time course of lipase activity (B), and the time course of protease activity (C) of intestinal wall homogenized with or without the lipase inhibitor orlistat (0.25 mg/ml final), then mixed 5:1 with 3 mg/ml elastase (0.5 mg/ml elastase final), and incubated at 37°C for 6 h (lipase activity was measured from an aliquot removed before mixing with elastase and diluted 10-fold). Values are means ± SD; $N = 5$ animals. Cell death was significantly reduced (*$P < 0.05$, paired $t$-test) with orlistat treatment, as was lipase activity (both digested all of the available fluorescent substrate, but the orlistat group took longer to do so); protease activity was not significantly different with or without orlistat.

Fig. 9. Cell death due to homogenates of intestinal segments that were previously incubated for 3 h at 37°C in a saline bath after luminal addition of saline, orlistat, aprotinin, or orlistat + aprotinin (or no fluid as control). Values are means ± SD; $N = 6$ animals. *$P < 0.05$ vs. no fluid; **$P < 0.05$ vs. no fluid or aprotinin (Tukey-Kramer HSD test).

Fig. 10. Cell death after 1-h exposure to homogenates of intestines from rats that underwent 90 min of splanchnic arterial occlusion (SAO) and 30 min of reperfusion, or a nonischemic sham surgery, mixed 19:1 with 200 mg/ml BSA or PBS for control (10 mg/ml BSA final). Values are means ± SD; $N = 4$ animals. *$P < 0.007$ vs. ischemic control (paired $t$-test vs. ischemic BSA, unpaired $t$-test vs. nonischemic control).
Surprisingly, in the nonischemic group, there was a three- to fourfold increase in total plasma FFAs (but only marginally significant; $P = 0.049$; with a minimum $P$ value of 0.025 for significance) over the 2-h period (Fig. 12). To determine whether this increase was due to the laparotomy, we additionally measured plasma FFAs at the same time points in two rats in which no laparotomy was performed and the intestine was not exteriorized. These rats exhibited the same increase in plasma FFA levels over time. If grouped with the other nonischemic animals, the difference becomes significant (not shown in figure), suggesting this increase in plasma FFA level is an effect of the anesthesia. In contrast, the ischemic animals with SAO shock experienced a significant decrease in their plasma FFA levels by 30 min of reperfusion.

In vivo shock treatments. To test whether a lipase inhibitor, on its own or in combination with a protease inhibitor, injected into the lumen of the intestine may provide protection during shock, we administered orlistat, aprotinin, as well as orlistat + aprotinin into the lumen of the rat small intestine and subjected the animals to a severe 2-h SAO followed by reperfusion. Orlistat on its own provided no protection for the animals compared with the saline group, as measured either by survival time (Fig. 13) or by gross morphological appearance of the intestine. While some animals were protected by aprotinin administration, others in that group showed no benefit from the protease treatment, and the overall increase in survival time was not significant. In contrast, the combined treatment with orlistat + aprotinin significantly increased the survival time of the animals and improved the gross morphological appearance of the intestine with fewer hemorrhagic lesions and less tissue swelling (Fig. 14).

**DISCUSSION**

This series of studies confirms that the intestine can be a major source of powerful cytotoxic mediators. We had previously shown that fresh homogenates of ischemic, but not control, intestines are highly cytotoxic and, similarly, that the wall of the intestine (even without digested food items in the

Fig. 11. Lipase activity vs. incubation time with fluorescent substrate of homogenates of ischemic intestine (90-min SAO, 30-min reperfusion) or nonischemic intestine, or intestinal wall homogenate digested with luminal fluid or PBS as control for 2 h at 37°C. Values are means ± SD; $N = 4$ animals. *$P < 0.05$ vs. intestinal wall + PBS.

Fig. 12. Concentration of free fatty acids (top) or protein (bottom) in plasma of ischemic or nonischemic sham controls, collected before ischemia (Pre) and after 90 min of ischemia and 30 min of reperfusion (R-30) (or after 2 h with the intestine exteriorized for nonischemic controls). Values are means ± SD; $N = 4$ animals. *$P < 0.02$ vs. nonischemic R-30 (nonpaired $t$-tests).

Fig. 13. Survival time after reperfusion of rats with saline, aprotinin, orlistat, or aprotinin and orlistat injected into the intestinal lumen and then subjected to 2 h of SAO followed by reperfusion for up to 3 h. Values are means ± SD; $N = 5$ animals. *$P < 0.014$ vs. saline group.
lumen of the intestine) becomes cytotoxic after entry of pancreatic enzymes (34). The results of the chloroform/methanol fractionation indicate that the cytotoxic factors are lipid in nature. The ability of a lipase inhibitor to decrease cell death supports the hypothesis that at least part of the cytotoxicity from the ischemic intestine is due to the products of lipase digestion, including FFAs.

FFAs are cytotoxic in a concentration-dependent manner via both apoptotic and necrotic mechanisms (3, 6, 13, 17, 18, 21, 24, 29). The necrotic mechanism may be due to the detergent action of the FFAs at high concentrations and appears to be receptor independent, although the molecular mechanisms remain to be clarified. The measured FFA concentrations in all wall homogenates were above the threshold for cytotoxicity (in the absence of lipid-binding proteins), supporting the hypothesis that they are the cause of the necrosis in the present experiments.

It has been shown that unsaturated fatty acids are more cytotoxic than saturated fatty acids and that, among the unsaturated fatty acids, arachidonic acid is more cytotoxic than linoleic, linolenic, or oleic acid (17). Composed of phospholipids, dietary fatty acids, and possibly de novo fatty acids as well, the total fatty acid composition of the intestinal homogenates in the rat is currently unknown. It is known that the fatty acid composition of the rat intestine phospholipids is 52% saturated, 33% polyunsaturated (one-third of which is arachidonic acid), and 15% monounsaturated fatty acids (almost entirely oleic acid) (42). As for dietary sources, the rats are fed the standard chow (Harlan Teklad Rodent Diet, W-8604) for which soybean oil is a major lipid source (www.teklad.com). Soybean oil triglycerides are primarily unsaturated, 51% linoleic, 23% oleic, 7% linolenic, with some saturated fatty acids as well, 10% palmitic, and 4% stearic. These data suggest that the majority of available fatty acids present in the rat homogenates (whether initially present before homogenization as FFAs, phospholipids, or triglycerides) are likely unsaturated, which may explain their high levels of cytotoxicity after protease digestion.

The high level of lipase activity we detected in the wall homogenate is likely one source of the FFAs in homogenates. It remains to be determined where exactly in the intestinal wall the lipase activity is present. It could be pancreatic lipase adhering to the luminal wall of the intestine and resistant to fluid rinsing (see MATERIALS AND METHODS), but this possibility is less likely. The lipase activity in the luminal fluid was comparatively low, and rinsing the intestine reduces the likelihood of pancreatic proteases to remain attached to the epithelium (34), so it is likely that the remaining pancreatic lipases are rinsed away as well. A more likely source may be intestinal lipase found in the epithelial cells of the intestinal mucosa. Intestinal lipase, also known as intestinal monoaoylglycerol lipase and intestinal glycerol-ester hydrolase, has a broad specificity and may belong to the carboxylesterase family (4). This form of lipase does not require a cofactor (7) and is also able to hydrolyze triglycerides, diglycerides, and even the monoglycerides, which pancreatic lipase cannot. That the lipase inhibitor orlistat was effective on its own “ex vivo” in preventing cell death (Fig. 9) and that orlistat in combination with the protease inhibitor aprotinin improved survival in a severe model of shock (Fig. 13) suggest that the enzyme does play a role in shock. However, the finding that lipase activity in homogenates of ischemic intestines was not greatly decreased compared with nonschematic intestines suggests that, even during ischemia, the majority of the activity remains in a separate compartment from the proteases (i.e., in cells as opposed to the interstitial space that the proteases would enter.

Fig. 14. Representative images of rat intestine treated with luminal injection of saline (top) or orlistat and aprotinin (bottom) after 2 h of SAO and 57 h (time of death in saline animal) or 3 h (end of observation time for orlistat + aprotinin animal) of reperfusion. Dark purple or black segments are locations of hemorrhage. The saline animal after reperfusion also has pale segments due to blood vessel drainage just before and after death.
during ischemia). Therefore, in the ex vivo case, the strong protection from orlistat may be in part because homogenization makes more lipase available than is available in vivo. The improved in vivo survival could also be due in whole or in part to orlistat’s inhibition of chymotrypsin. Thus it remains to be determined just how important lipase activity is in shock. Whatever the lipase activity, FFAs are present in the intestinal lumen within digested food and in the intestinal wall as part of normal lipid digestion. They also get carried into the intestine while bound to albumin. The important factor that determines their cytotoxicity is the level of bound vs. unbound FFAs in the intestine (see below).

It is interesting that we detected lower levels of FFAs in chymotrypsin digests than in either the undigested homogenates or digests generated with the mixture of pancreatic enzymes in the luminal fluid (Fig. 6A). The fact that the chymotrypsin digest contained less FFA than the controls may have been the consequence of digestion of lipase by chymotrypsin. However, if that were the only factor, we would expect the luminal fluid digest with its greater protease activity to have even less FFA. Instead, the luminal fluid digest had significantly more FFA than the chymotrypsin digest. The luminal fluid alone has very little FFA, suggesting that the luminal fluid must contribute to FFA in the luminal fluid-digested homogenate via addition of either lipase or lipase substrate. A possible explanation is that luminal fluid may provide enough pancreatic lipase activity to hydrolyze additional FFA before being digested by proteases.

The results of the present fractionation study also demonstrate that the generation of cytotoxic lipids in the wall of an ischemic intestine may not be sufficient to cause cell death. There is a highly protective component in the protein fractions of the intestinal wall that must also be eliminated in order for lipid cytotoxicity to take effect. The exact identity of the protein(s) responsible for this protection here remains to be determined. Any lipid binding protein could act as protective protein. An example is the intestinal fatty acid binding protein, which is released during intestinal ischemia (20). But a most ubiquitous FFA binding protein in the circulation is albumin, with an average concentration of 48.9 mg/ml in plasma and ranging from −7 mg/ml in the interstitial space of adipose tissue to 13 mg/ml in the interstitial space of skeletal muscle (8). As a lipid-binding protein (14, 38), albumin is capable of preventing cell death (18), as was observed here already at 5 mg/ml with both bovine and rat serum albumin.

Albumin predigested by proteases, however, does not reduce the FFA cytotoxicity. Therefore, our results suggest that a major component of the protection against lipid toxicity may be due to albumin. Besides the role of albumin as a protein responsible for maintaining osmotic homeostasis, its lipid-binding property may be of major importance in minimizing FFA-mediated toxicity. Since there is no difficulty to solubilize oleic acid at cytotoxic concentrations and since FFAs do not appear to precipitate out of solution in protease-digested homogenates, we suggest that the lipid-binding property of albumin in the plasma is not for the purpose of increasing FFA solubility, but for keeping the FFA sequestered.

Beyond the conditions provided by stasis and an ischemic state, the presence of cytotoxic factors within the gut may be responsible for the intestinal necrosis observed in shock. As yet, it is still uncertain how intestinal ischemia affects the function of other organs in shock, although there are now a number of possibilities. Cytotoxic activity has been measured in the mesenteric lymph of animals in shock (19), suggesting that FFAs could be returned to the central circulation upon reperfusion. This could result in direct cytotoxicity, such as that described here, or in the “lipotoxicity” that is the result of elevated plasma FFA levels, e.g., in diabetes and other diseases (21, 41).

It is not known why, in the animal model used here, total (i.e., both bound and unbound) FFA levels decreased in the plasma during shock. This could be due to the clamping of the major lymphatic vessel exiting the intestine during ischemia, cutting off the normal FFA supply to the main circulation. The ratio of bound to unbound fatty acids also is not known. The concentration of unbound FFAs could increase in shock, even with total FFAs decreasing. Alternatively, unbound FFAs may preferentially be transported into cell membranes and leave the circulation. It is of interest that, in porcine hemorrhagic shock, we found significantly elevated levels of total FFA in the plasma (unpublished results), suggesting that measured levels in plasma may vary greatly with the model used.

Regardless, it is not necessary for FFAs to leave the intestine to affect peripheral organs in shock. Necrosis within the intestine could result in fluid loss to the gut on reperfusion, which may contribute to blood pressure reduction and underperfusion (28). Intestinal necrosis also causes release of inflammatory mediators (1, 10) and phospholipids (especially phosphotidyl serine and phosphotidyl ethanolamine) that, on reperfusion, could cause the global inflammation and disseminated intravascular coagulation, respectively (15).

In our ex vivo study, we observed considerable variability in the cytotoxicity of homogenized intestinal segments in the “no fluid” or luminally added “saline” groups. This may be due to variations in the amount and location of luminal content. The length of time between onset of shock and the last ingestion of food is known to affect the outcome of shock (1), possibly by affecting the location and concentration of pancreatic proteases in the lumen. We observed that intestines with less luminal content tended to have lower levels of cytotoxicity.

In addition to variability within a model, we expect variations from model to model in such areas as the kinetics of albumin entering the intestinal interstitium, protease digestion of albumin, and exodus of FFAs or other inflammatory or shock mediators, as well as the FFA concentrations in plasma as mentioned above. Thus, for example, in a hemorrhagic shock model, it is key to determine whether new albumin enters at a faster or slower rate than its proteolytic degradation. Compared with an arterial occlusion model, depletion of intact albumin concentrations may take longer in a hemorrhagic shock model, but, by the same token, FFAs or other shock mediators could enter the main circulation much earlier.

In the present in vivo arterial occlusion model, neither orlistat nor aprotinin alone could significantly improve survival, while together they significantly improved survival. Pilot studies showed that neither orlistat nor aprotinin stimulated pseudopod production in neutrophils. Nor did these enzymes prevent pseudopod formation after N-formyl-methionyl-leucyl-phenylalanine stimulation, although there appeared to be slightly less formation in the presence of orlistat. Therefore, it is likely that orlistat and aprotinin accomplished protection via reduction in formation or release of shock factors. Al-

H1790 INTESTINAL AUTODIGESTION AND CYTOTOXICITY

AJP-Heart Circ Physiol • VOL 294 • APRIL 2008 • www.ajpheart.org
though there is an incomplete proof due to orlistat’s partial inhibition of chymotrypsin, the necessity of orlistat to achieve protection suggests that these shock factors include FFAs.

We have seen repeatedly that serine protease blockade in the lumen of the intestine improves shock outcome and reduces release of inflammatory mediators (11, 30, 39). The determination of whether protection is granted in part by prevention of lipid-binding protein digestion, i.e., FFA release, may require the development of a method to neutralize or eliminate excess unbound FFA in the intestine and central circulation. In pilot studies, we determined that albumin added to aprotinin reduces its effectiveness as a protease inhibitor, and thus it was not included in the luminal treatments in our in vivo study. We are, however, presently investigating the effectiveness of intravenous albumin supplementation as a replacement for, or in combination with, luminal enzyme inhibition in shock prevention. We are also evaluating other possible lipase inhibitors for biocompatibility, effectiveness, and cross reactivity with proteases.

In the SAO model of shock, besides total ischemia in the small intestine, a partial ischemia of the pancreas occurs. Thus the possibility exists that many of the same mediators formed in the intestine could be formed in the pancreas, if ischemia activates the zymogen proteases. We have shown previously that homogenates of pancreas, as well as lipase- and possibly protease-digested homogenates of other organs besides the intestine (liver, etc.), can be cytotoxic (43). Therefore, further studies are needed to investigate the intravenous use of protease and/or lipase inhibitors.

In conclusion, intestinal wall homogenates contain, in part as a result of endogenous lipase activity, cytotoxic levels of FFAs that are bound to protective proteins. If those proteins are also digested by protease, fatty acids are free to cause necrosis.

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DISCLAIMER
The authors declare no financial conflict of interest with any corporations marketing the agents used in this study.

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