The American Journal of Pathology, Vol. 180, No. 1, January 2012

DOI: 10.1016/j.ajpath.2011.09.031

Gastrointestinal, Hepatobiliary, and Pancreatic Pathology

Serine Protease Inhibition Reduces Post-Ischemic Granulocyte Recruitment in Mouse Intestine

Thomas Gobbetti,*†‡§ Nicolas Cenac,*†‡ Jean-Paul Motta,*†‡ Corinne Rolland,*†‡ Laurence Martin,*†‡ Patricia Andrade-Gordon,¶ Martin Steinhoff,‖ Elisabetta Barocelli,§ and Nathalie Vergnolle*†‡**

From INSERM, U1043, CNRS,† U5282, and the Université de Toulouse,‡ UPS, Centre de Physiopathologie de Toulouse Purpan (CPTP), Toulouse, France; the Department of Pharmacological, Biological and Applied Chemical Sciences,§ University of Parma, Parma, Italy; RW Johnson & Johnson,¶ Spring House, Pennsylvania; the Departments of Dermatology and Surgery,‖ University of California San Francisco, San Francisco, California; and the Department of Physiology and Pharmacology,** University of Calgary, Calgary, Alberta, Canada*

Proteases and proteinase-activated receptor (PAR) activation are involved in several intestinal inflammatory conditions. We hypothesized that serine proteases and PAR activation could also modulate the intestinal injury induced by ischemia-reperfusion (I-R). C57Bl/6 mice were subjected to 90 minutes of intestinal ischemia followed or not by reperfusion. Sham-operated animals served as controls. After ischemia, plasma and tissue serine protease activity levels were increased compared to the activity measured in plasma and tissues from sham-operated mice. This increase was maintained or further enhanced after 2 and 5 hours of reperfusion, respectively. Trypsin (25 kDa) was detected in tissues both after ischemia and 2 hours of reperfusion. Treatment with FUT-175 (10 mg/kg), a potent serine protease inhibitor, increased survival after I-R, inhibited tissue protease activity, and significantly decreased intestinal myeloperoxidase (MPO) activity and chemokine and adhesion molecule expression. We investigated whether serine proteases modulate granulocyte recruitment by a PAR-dependent mechanism. MPO levels and adhesion molecule expression were significantly reduced in I-R groups pre-treated with the PAR_1 antagonist SCH-79797 (5 mg/kg) and in $Par_2^{-/-}$ mice, compared, respectively, to vehicle-treated group and wild-type littermates. Thus, increased proteolytic activity and PAR activation play a pathogenic role in intestinal I-R injury. Inhibition of PAR-activating serine proteases could be beneficial to reduce post-ischemic intestinal inflammation. ***(Am J Pathol 2012, 180:141–152; DOI: 10.1016/j.ajpath.2011.09.031)***

Acute mesenteric ischemia is a potentially fatal abdominal emergency implicated in a large array of pathological conditions that involve a critical reduction of blood flow to the gut as consequence of surgical states (abdominal aortic aneurism surgery, cardiopulmonary bypass), vessel occlusions (embolism or thrombosis), septic or hemodynamic shock, intestinal hernias, and also transplants of the small intestine.[1–3] Although acute mesenteric ischemia accounts for only 1% to 2% of gastrointestinal illnesses, it still causes a high in-hospital mortality rate (60% to 80%).[3]

The pathogenesis of ischemic injury is related to the interruption of blood supply to the gut, which results in rapid metabolic damage to active tissues, particularly to the labile cells of mucosa. Paradoxically, reoxygenation, depending on the time and intensity of the ischemia, further increases the damage, causing an additional cell injury known as reperfusion injury. This acute inflammatory response causes a rapid deterioration of the intestinal barrier, leading to intestinal bacterial translocation through the epithelial mucosa to extra-intestinal sites (mesenteric lymph nodes, liver, and spleen).[3,4] The subsequent sepsis and production of proinflammatory molecules leads to the development of a systemic inflammatory response syndrome, which can progress to multiple organ failure involving organs such as liver, heart, kidneys, and lungs.[2,5,6]

Proteases, particularly serine proteases, act as signaling molecules that are able to send specific signals to cells involved in intestinal inflammatory responses through the activation of a subclass of four G protein–

Supported by INSERM-Avenir program, Agence Nationale de la Recherche, Fondation Bettencourt-Schueller, and Fondation Schlumberger (N.V.).

Accepted for publication September 20, 2011.

Address reprint requests to Nathalie Vergnolle, Ph.D., INSERM U1043, CHU Purpan, BP3028, 31024 Toulouse-Cedex, France, Tel: (+33) 562744500. E-mail: nathalie.vergnolle@inserm.fr.

coupled proteinase-activated receptors (PAR_1, PAR_2, PAR_3, and PAR_4).[7–12] To activate those receptors, which are largely expressed in the gut, proteases cleave at specific sites within the extracellular N-terminus domain of PARs. This cleavage unmasks a new N-terminal sequence that acts as a tethered ligand, which binds to the receptor to initiate multiple signaling cascades.[8] Several findings report that direct injection of proteases such as thrombin, trypsin, tryptase, or selective agonist for PAR_1 and PAR_2 into the paw of rodents produces inflammation.[13–15] In addition, we have demonstrated that luminal administration of selective peptides, agonists for PAR_1, PAR_2, and PAR_4 provoked a colonic inflammatory response.[16–21]

All these observations provide the background for the hypothesis that proteases and PARs could play a major role in the pathogenesis of intestinal inflammation associated with ischemia-reperfusion (I-R) injury. Although a role for proteases in ischemic tissues has been suggested in kidney,[22] liver,[23,24] heart,[25,26] and digestive tract,[27,28] it is not clear what role proteases may play in ischemic disorders of the intestine, which is, above all, the organ the most exposed to a variety of proteases.

We used a model of small intestine ischemia developed in mice by reversible occlusion of the superior mesenteric artery for 90 minutes, followed by 0, 2, or 5 hours of reperfusion. By combining pharmacological and gene-deletion approaches, we demonstrate here the pivotal role of serine proteases, through the activation of PAR_1 and PAR_2, in inflammatory damage associated with intestinal ischemia-reperfusion.

Materials and Methods

Animals

C57Bl/6 male mice (8 weeks old) were obtained from Janvier (Le Genest Saint Isle, France). $PAR_2^{-/-}$ and wild-type littermates ($Par_2^{+/+}$) were originally provided by Johnson & Johnson Pharmaceutical Research Institute.[29,30] Animals were kept under pathogen-free conditions and were given free access to food and water. All of the experimental protocols were approved by local animal care and ethics committees and followed the guidelines of French Councils on Animal Care.

Surgical Procedure and Treatments

Mice were anesthetized with sodium pentobarbital (Pentobarbital sodique, Ceva Santé Animale, Libourne, France) (50 mg/kg intraperitoneally). After abdominal laparotomy, the small bowel was retracted to the left and the superior mesenteric artery was temporarily occluded using a microvascular clip to cause ischemia. After 90 minutes, the clip was gently removed, allowing reperfusion. Mice were sacrificed right after the ischemic period (time 0), 2, or 5 hours after reperfusion. Sham-operated (SO) animals, in which abdominal laparotomy and artery isolation were performed without occlusion of the vessel, served as controls. After the surgical procedure, the midline incision of the abdominal wall was closed by two-layer sutures. After recovering from anesthesia, animals were returned to their cages. At the end of the experiments, animals were anesthetized with sodium pentobarbital to collect blood through cardiac puncture. Euthanasia was performed by cervical dislocation. Heparinized blood samples were centrifuged at 7500 × *g* for 10 minutes to obtain plasma. For biochemical analysis, ileal tissue was excised and processed.

Mice were treated as follows: the serine protease inhibitor FUT-175 (10 mg/kg dissolved in saline; Santa Cruz Biotechnology, tebu-bio, Le Perray en Yvelines Cedex, France) was administered intravenously at the beginning of ischemia and repeated at the moment of reperfusion; the PAR_1 antagonist SCH-79797 (5 mg/kg dissolved in carboxymethylcellulose; Tocris Bioscience, Bristol, UK) was administered intraperitoneally twice, at 18 hours and 2 hours before the surgery. A similar surgical procedure was used for both the $Par_2^{+/+}$ and $Par_2^{-/-}$ mice.

Protease Activity Assay

Trypsin-like proteolytic activity was measured both in intestinal tissue and plasma using a microplate reader NOVOstar (BMG Labtech, Champigny s/Marne, France). On sacrifice, plasma samples were collected and a piece of ileal tissue was excised and rinsed in 1× PBS to remove all intraluminal content and was then homogenized in 1 mL of 1× PBS (pH 7.2) with 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS using Precellys 24 homogenizer in Precellys lysing CK14 tubes (Bertin Technologies, Ozyme, France). The homogenized tissues were centrifuged at 5000 × *g* for 5 minutes. The trypsin-like activity was measured using tosyl-Gly-Pro-Arg *p*-nitroanilide (150 μmol/L; Sigma, Saint Quentin Fallavier, France) as substrate in 100 mmol/L Tris/HCl, 1 mmol/L $CaCl_2$ buffer (pH 8). The hydrolysis rate was measured at 37°C over a 30-minute period in absorbance at 405 nm.[31] Activity was standardized to the rate generated by trypsin of known concentration from porcine pancreas (Sigma). Plasma activity was expressed as U/mL, whereas tissue activity was normalized to the homogenate protein concentrations determined with a BCA kit (Pierce, Thermo Scientific, Courtaboeuf, France) and expressed as U/mg protein. When required, protease inhibitors were added to tissue supernatants or plasma samples: E-64 10 μmol/L (tebu-bio), Pepstatin A 1 μmol/L (Sigma), AESBF 10 mmol/L (tebu-bio), FUT-175 50 μmol/L (tebu-bio). Protease inhibitor doses were chosen according to previous data reported in the literature.[32–37]

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was measured as an index of granulocyte infiltration as previously described in ileal tissues harvested at the time of sacrifice.[38] Briefly, ileal tissue samples were homogenized in a solution of 0.5% hexadecyltrimethylammonium bromide dissolved in phosphate buffer solution (pH 6) using Precellys 24 homogenizer in Precellys lysing CK14 tubes (Bertin Technologies). The homogenized tissues were centrifuged at 13,000 × *g* for 5 minutes (4°C), and the supernatants