

Generation of *in vivo* activating factors in the ischemic intestine by pancreatic enzymes

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One of the early events in physiological shock is the generation of activators for leukocytes, endothelial cells, and other cells in the cardiovascular system. The mechanism by which these activators are produced has remained unresolved. We examine here the hypothesis that pancreatic digestive enzymes in the ischemic intestine may be involved in the generation of activators during intestinal ischemia. The lumen of the small intestine of rats was continuously perfused with saline containing a broadly acting pancreatic enzyme inhibitor (6-amidino-2-naphthyl *p*-guanidino-benzoate dimethanesulfate, 0.37 mM) before and during ischemia of the small intestine by splanchnic artery occlusion. This procedure inhibited activation of circulating leukocytes during occlusion and reperfusion. It also prevented the appearance of activators in portal venous and systemic artery plasma and attenuated initiating symptoms of multiple organ injury in shock. Intestinal tissue produces only low levels of activators in the absence of pancreatic enzymes, whereas in the presence of enzymes, activators are produced in a concentration- and time-dependent fashion. The results indicate that pancreatic digestive enzymes in the ischemic intestine serve as an important source for cell activation and inflammation, as well as multiple organ failure.

rat | splanchnic arterial occlusion | shock | multiple organ failure | microcirculation

Shock is a life-threatening cardiovascular complication (1). Cellular activation in the circulation is a relatively early event in shock that can be detected by leukocyte or endothelial superoxide production, pseudopod projection, expression of membrane adhesion molecules, and many other cell functions (2, 3). Cell activation fundamentally alters the biomechanics of microvascular blood flow by a shift in rheological, adhesive, and cytotoxic cell properties. The interaction between activated leukocytes and endothelial cells is followed by cell and organ failure (4). The level of activation correlates with survival after the shock (5), but the mechanism for production and the source of activating factors has remained an unresolved problem. Several candidates have been proposed, including endotoxins, lipid-derived products, and cytokines (6–9).

Recently, we have demonstrated that the supernatant of the homogenized pancreas, but less so homogenates of other organs, mediates a powerful activation of cardiovascular cells (10). Incubation of homogenates from nonactivating organs, such as the liver or intestine, with low concentration of serine proteases also increases the ability to activate leukocytes. An enzyme inhibitor could block the activation, suggesting that pancreatic enzymes may play a central role in the production of activating factors.

Pancreatic enzymes are discharged via the pancreatic duct into the duodenum and intestine as a requirement for digestion. We hypothesize that pancreatic enzymes in the ischemic intestine may be involved in the production of activating factors for circulatory cells in shock. Ischemia in the intestine may cause an increased epithelial and endothelial permeability (11) and allow such activators to enter the systemic circulation via the portal vein and/or intestinal lymphatics (12, 13). Thus, after entering the systemic circulation, digestive enzymes and their products

may play a central role in activation of leukocytes or endothelial cells in shock. The pathophysiological role of pancreatic enzymes in intestinal ischemia has not been elucidated.

The objective of this study was to investigate the influence of digestive enzymes on activation of circulating leukocytes during shock produced by splanchnic arterial occlusion. A broad-acting enzyme inhibitor placed into the intestinal lumen served to prevent the formation of central activation and initiation of multiple organ failure in shock.

Materials and Methods

Splanchnic Arterial Occlusion After Pancreatic Ligation. Male Wistar rats (290–340 g; Charles River Breeding Laboratories) were maintained on a standard rat chow and water ad libitum. All experiments were reviewed and approved by the University of California San Diego Animal Use and Care Committee. After general anesthesia (sodium pentobarbital, 25 mg/kg, i.p.), the left femoral artery was cannulated with polyethylene tubing (PE-50, Clay Adams) to record arterial pressure. The left femoral vein was cannulated (PE-50, Clay Adams) for administration of anesthetic agent. The celiac and superior mesenteric artery were isolated through an abdominal midline incision and looped with silk thread (3-0) at their aortic origin. The threads were guided out of the abdomen through polyethylene tubing (PE-50), and arteries were occluded by application of tension to the threads.

In the first experimental sequences, three groups were formed: I, a nonischemic sham shock ($n = 5$ rats); II, a splanchnic artery occlusion shock ($n = 10$); and III, a splanchnic artery occlusion shock group with pancreatic vessel ligation ($n = 10$). In group III, all pancreatic arteries and draining veins were ligated before splanchnic ischemia. Considering that the rat pancreas consists of three main parts, the splenic, duodenal, and gastric gland, the pancreatic ligation was achieved as follows. The communication between splenic and duodenal gland was dissected to expose the origin of the splenic artery or vein from the celiac artery or the superior mesenteric vein. The splenic artery and vein were ligated at their origin. Short gastric arteries and veins and other small communicators from the stomach and spleen were also ligated (silk 4-0) to isolate the splenic gland from the systemic circulation. To isolate the blood supply to the duodenal gland, the vessels connecting along the superior mesenteric vein and also the duodenum were ligated, because there are small anastomoses that originate from the marginal artery and vein of the duodenum. The duodenum with its marginal artery was ligated at the junction to the stomach and the jejunum. Isolation of blood supply to the pancreas was confirmed by central injection of heparinized and filtered India ink (30% in saline) and detailed examination of a color changes in the surrounding tissue but not in the pancreas *per se*.

Abbreviations: IF, intestinal fluid; SAL, saline; ANG2, 6-amidino-2-naphthyl *p*-guanidino-benzoate dimethanesulfate.

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All groups except the sham group were subjected to 100 min of splanchnic arterial occlusion and reperfused for 30–120 min. Thirty minutes after reperfusion, arterial blood samples in heparin were collected from five rats in each group. To separate plasma, the blood samples were immediately centrifuged.

Activation of Naive Leukocytes by Experimental Plasma. Because activated leukocytes have high probability to become trapped in the microcirculation, there is a need to test the ability of plasma to activate with cells other than autologous circulating cells. Therefore, the activation produced by rat plasma aliquots was tested on naive granulocytes derived from nonischemic controls. Human leukocytes from laboratory volunteers without symptoms were used to minimize further use of donor animals. Pilot studies had shown that the levels of activation achieved by rat plasma applied to rat and to human granulocytes were not significantly different (10). Granulocyte-rich plasma was collected after about 40-min sedimentation of 40 ml of venous blood from a healthy human volunteer, layered onto 3.5 ml of Histopaque (Sigma), and centrifuged ($600 \times g$, 20 min). The leukocytes and erythrocytes (about 1 ml) were suspended in 1 ml of Krebs–Henseleit solution layered onto 2.5 ml of 55% and 74% isotonic Percoll solution (Sigma). After centrifugation at $600 \times g$ for 15 min, the intermediate purified granulocyte layer was removed and resuspended in 1 ml of 10 mM phosphate-buffered saline. These control cells are referred to as naive granulocytes.

The ability of plasma from the rats with splanchnic arterial occlusion to activate was determined by pseudopod formation on naive granulocytes. Suspended granulocytes ($100 \mu\text{l}$; about $10,000 \text{ per mm}^3$ in phosphate-buffered saline) were mixed with $100 \mu\text{l}$ of test plasma from shock rats. This mixture was incubated for 10 min at room temperature. Glutaraldehyde in phosphate-buffered saline (3%, $100 \mu\text{l}$) was added to arrest the pseudopods. Granulocytes with cytoplasmic projections (pseudopodia) $>1 \mu\text{m}$ were designated as activated cells. The fraction of activated granulocytes, of at least 200 cells, was counted.

Splanchnic Arterial Occlusion with Blockade of Pancreatic Enzymes in the Intestine. In this second set of experiments, the pancreatic blood supply was left intact, and instead the intestinal lumen was rinsed and portal venous blood samples were collected according to the following procedure. Besides femoral artery and vein catheters, a line (PE-50) was inserted into a cecal branch of the portal vein for blood collection. Because venous congestion was frequently encountered, the cecum was removed before cannulation of the portal vein.

The proximal duodenum and terminal ileum were cannulated (PE-280), and the initial intestinal contents were gently rinsed with 30 ml of saline and collected. Thereafter the intestinal lumen was gently rinsed with 3,000 ml of saline (37°C) at constant pressure (10–15 mmHg; 1 mmHg = 133 Pa) and then discarded. A closed-loop intestinal perfusion from the duodenum to the terminal ileum was set up with a peristaltic pump (MasterFlex, Cole–Parmer) with a priming volume of 50 ml.

Experimental Procedure. The intestinal contents were mixed and centrifuged ($500 \times g$, 10 min). The supernatant is referred to as intestinal fluid (IF). The small intestinal lumen was perfused at constant flow rate (4.0 ml/min) with the following three solutions: (i) 45 ml of diluted IF with 5 ml of 5% glucose (IF group, $n = 5$ rats); 45 ml of saline (ii) without, and (iii) with 10 mg of 6-amidino-2-naphthyl *p*-guanidinobenzoate dimethanesulfate (ANGD, 0.37 mM, Nafamostat Mesilate, Torii Pharmaceutical, Chiba, Japan) (14) in 5 ml of 5% glucose (SAL and ANGD groups, $n = 5$ rats, respectively). Five nontreated animals were used as nonischemic controls.

After 15 min of intestinal perfusion, the animals were subjected to 100 min of splanchnic ischemia, which was confirmed

by cyanotic organ discoloration and the loss of pressure pulsation in the mesentery. After 100 min of splanchnic ischemia, the celiac and superior mesenteric arteries were reperfused. One milliliter of circulating fluid in the intestine was collected from the reservoir before ischemia and 90 min after ischemia. Arterial and portal venous blood (0.3 ml each) was sampled before ischemia, after 90 min of ischemia, as well as 30, 60, and 120 min of reperfusion. Plasma Lyte A (0.6 ml; Baxter Scientific Products, McGaw Park, IL) was administered (i.v.) immediately after each blood withdrawal. Twenty microliters of arterial blood was used to measure the fraction of granulocytes with pseudopods in the circulation, and $20 \mu\text{l}$ was used for leukocyte counts. The remainder of the arterial and portal venous blood aliquot was centrifuged at $1,500 \times g$ for 10 min, and supernatant plasma was stored at -70°C . After centrifugation, the blood cells were resuspended in 0.6 ml of Plasma Lyte A and reinjected (i.v.).

Leukocyte Counts and Activation. Leukocyte counts were made with a hemocytometer. Immediately after withdrawal, $20 \mu\text{l}$ of arterial blood was fixed in 3% glutaraldehyde (Fisher Scientific; in 10 mM phosphate-buffered saline), and stained with $20 \mu\text{l}$ of 0.02% crystal violet in phosphate-buffered saline. The fraction of activated granulocytes was counted (see above).

Organ Leukocyte Infiltration. Myeloperoxidase activity was used as marker for assessment of leukocyte infiltration into the small intestine (about 20 cm proximal to the ileocecal junction, 3 cm in length), liver, and right lung. Tissue myeloperoxidase levels were determined by a spectrophotometric method (15). Myeloperoxidase from human purulent leukocytes (Sigma) served as standard.

Protease Activity in Intestinal Fluid. Serine protease activity of any of the intestinal lumen perfusates was determined by a spectrofluorometric method (16). Trypsin (1440 BAEE units/mg, Sigma) was used as standard.

Intestinal Histology. A sample of small intestine was longitudinally dissected, fixed in 10% buffered formalin, and embedded in paraffin. Five-micrometer sections were made, stained with hematoxylin and eosin, and examined at $\times 200$ magnification. Severity of intestinal injury was estimated by the length between the tip of the villi and the musculus mucosae, a measure of the mucosal layer thickness (17). In each specimen, the measurement was made at 10 randomly selected locations and averaged.

Bile Flow Rate. Bile flow rate was determined to indicate the severity of liver injury (18). The bile flow rate for 15 min was measured at each time point before splanchnic arterial occlusion and during reperfusion, and normalized for each animal with the flow rate before ischemia.

Lung Wet/Dry Weight Ratio. The left lung lobes were harvested, wet weight was determined, and samples were dried at 70°C for 72 hr. The residuum was weighed, and the ratio of wet to dried weight was computed.

In Vitro Production of Activator. In three rats, the intestine was removed and homogenized in 0.25 mM sucrose solution at 1:5 weight ratio and centrifuged ($1,000 \times g$; 10 min). The sediment was discarded, and the supernatant was ultracentrifuged ($15,900 \times g$; 30 min; 4°C). The supernatant intestinal homogenate was mixed with 0, 1, 5, and 10 mg/ml trypsin (Sigma) and incubated for 0, 30, 60, 90, 120, and 150 min. At each time, the ability to activate naive leukocytes was tested by pseudopod formation (see above). Each test was completed in quadruplicate.

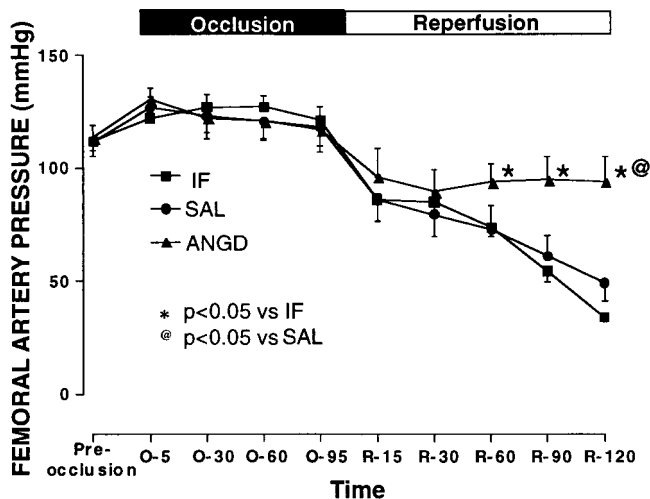


Fig. 1. Time course of femoral mean arterial blood pressure. Numbers on the abscissa indicate the minutes after splanchnic arterial occlusion (O) and reperfusion (R). Squares refer to the intestinal fluid (IF) group, circles the saline rinse (SAL) group, and triangles to the ANGD group.

Statistics. Measurements are expressed as mean \pm standard error of mean. Statistical significance in wet/dry ratio of the lung, tissue myeloperoxidase level, and histological measurements were compared by Fisher's protected least-squares difference. Values of mean arterial blood pressure, bile flow rate, activation of circulating leukocytes, serine protease activity in the intestinal fluid, and activation produced by plasma, and *in vitro* experimental data were tested by the analysis of variance with Bonferroni's correction. $P < 0.05$ was considered significant.

Results

Pancreatic Vascular Ligation in Splanchnic Artery Occlusion Shock.

The femoral blood pressure during the occlusion period was not significantly different between group II without and group III with pancreatic vessel ligation, respectively. Thirty minutes after reperfusion, the arterial pressure dropped in both groups. Although the mean arterial pressure fell slightly less in the ligated group (63 ± 17 mmHg in group II vs. 47 ± 8 mmHg in group III with pancreatic ligation, $P > 0.05$), 60 min after reperfusion both groups reached 100% mortality. The ability of arterial plasma to activate naive leukocytes is significantly elevated 30 min after reperfusion in these groups, when compared with sham shock group I ($22.6 \pm 4\%$ in splanchnic arterial occlusion group II and $5.3 \pm 2\%$ in sham shock group I) ($P < 0.05$). This sequence of events is unaffected by pancreatic vessel ligation ($22.7 \pm 5\%$ in group III), indicating that the pancreas is less likely the predominant source of a leukocyte activator. Instead, the pancreas may serve as a source of enzymes that are discharged into the intestine as part of normal digestion. Therefore, our remaining study is focused on pancreatic enzymes in the intestine.

Significance of Proteolytic Reactions in the Ischemic Intestine. Mean arterial pressure.

After splanchnic arterial occlusion, all animals exhibited a slight increase in mean arterial pressure and a sudden decrease after the reperfusion (Fig. 1). The groups with intestinal fluid in the small intestine (IF) and with a saline rinse of the intestinal lumen (SAL) did not recover from the hypotension after the reperfusion. In contrast, the mean artery pressure in the rats with saline rinse and ANGD in the intestinal lumen was significantly higher after reperfusion ($P < 0.01$ ANGD vs. IF and SAL groups). The mean arterial pressure in the ANGD group

remained on average at 84% of its initial value after 2 hr of reperfusion.

Serine protease activity in the ischemic intestine. Before splanchnic ischemia, the serine protease activity in the intestinal fluid in the IF group was $5.8 \pm 1.8 \times 10^3$ units/ml. Intestinal lavage with saline served to lower the activity to $2.0 \pm 0.9 \times 10^3$ units/ml ($P < 0.05$, IF vs. SAL group) and ANGD further decreased the activity to $1.2 \pm 0.1 \times 10^3$ units/ml ($P < 0.05$, SAL vs. ANGD). After 90 min of ischemia, the serine protease activity in the intestinal fluid of the IF, SAL, and ANGD groups was $5.6 \pm 2.3 \times 10^3$, $3.3 \pm 2.3 \times 10^3$, and $1.2 \pm 0.2 \times 10^3$ units/ml, respectively ($P < 0.05$, SAL vs. ANGD). Intestinal perfusion with ANGD kept the serine protease activity lower than in other groups ($P < 0.01$ ANGD vs. IF and SAL groups).

Leukocyte count and activation. In the preischemic period, the numbers of leukocytes in the arterial blood were the same (5.9 ± 0.4 per mm^3 in the IF group, 5.4 ± 0.3 per mm^3 in the SAL group, and 5.5 ± 0.2 per mm^3 in the ANGD group). In the IF and SAL groups, the leukocyte count in arterial blood started to decrease after ischemia and reached its lowest value 120 min after reperfusion. Intestinal perfusion of protease inhibitor completely ameliorated the leukopenia (Fig. 2A).

Although no differences were detected among groups in the preischemic period (Fig. 2A), splanchnic ischemia and reperfusion led to an increased number of leukocytes with pseudopod projections. Intestinal perfusion of protease inhibitor, however, served to significantly lower the number of activated cells.

Activation potential of shock plasma. Arterial and portal venous plasma from nonischemic control animals only mildly activated naive leukocytes ($8.0 \pm 3.1\%$ and $9.0 \pm 2.0\%$, respectively). In the preocclusion period, the ability to activate naive leukocytes with arterial or portal venous plasma from either of the groups did not differ from the one in the plasma of nonischemic controls (Fig. 2B).

Ninety minutes of splanchnic ischemia served to increase the activation produced by portal venous plasma in the IF and SAL groups ($P < 0.01$ vs. nonischemic control, data not shown). At this time point, the portal venous plasma in both IF and SAL groups yielded higher activation values than did arterial plasma ($P < 0.01$). In the ANGD group, however, the activation produced by either the arterial or portal venous plasma was not significantly increased throughout the experiment, and remained much lower than in either the IF or SAL group ($P < 0.01$) (Fig. 2B).

Tissue myeloperoxidase levels. At 120 min after reperfusion, the intestinal, hepatic, and pulmonary myeloperoxidase levels were increased in all organs. This is an early indicator of leukocyte infiltration and organ failure in shock (19). Intestinal perfusion of protease inhibitor, however, significantly attenuated the myeloperoxidase activity (Fig. 3A-C).

Intestinal injury after splanchnic ischemia reperfusion. Splanchnic arterial occlusion and reperfusion led to a reduction of the mucosal thickness, a phenomenon associated with morphological damage to the intestine. Intestinal perfusion of protease inhibitor served to maintain mucosal thickness and to reduce intestinal injury (Fig. 3D).

Bile flow rate. The baseline values for the bile flow rate were not different among groups (95 ± 12 $\mu\text{l}/\text{min}$ in IF group, 75 ± 9 $\mu\text{l}/\text{min}$ in SAL group, 85 ± 7 $\mu\text{l}/\text{min}$ in ANGD group). Splanchnic arterial occlusion caused a reduction of bile production without significant differences among groups. A small degree of recovery was observed after reperfusion in the IF and SAL groups. Intestinal perfusion of protease inhibitor increased the recovery of bile flow rate (Fig. 3E).

Pulmonary wet/dry ratio. The level of edematous lung injury after splanchnic arterial occlusion and reperfusion is greatly increased in the IF and SAL groups. Perfusion of the intestinal lumen with protease inhibitor served to reduce the average lung wet/dry ratio to the level of the nonischemic control (Fig. 3F).

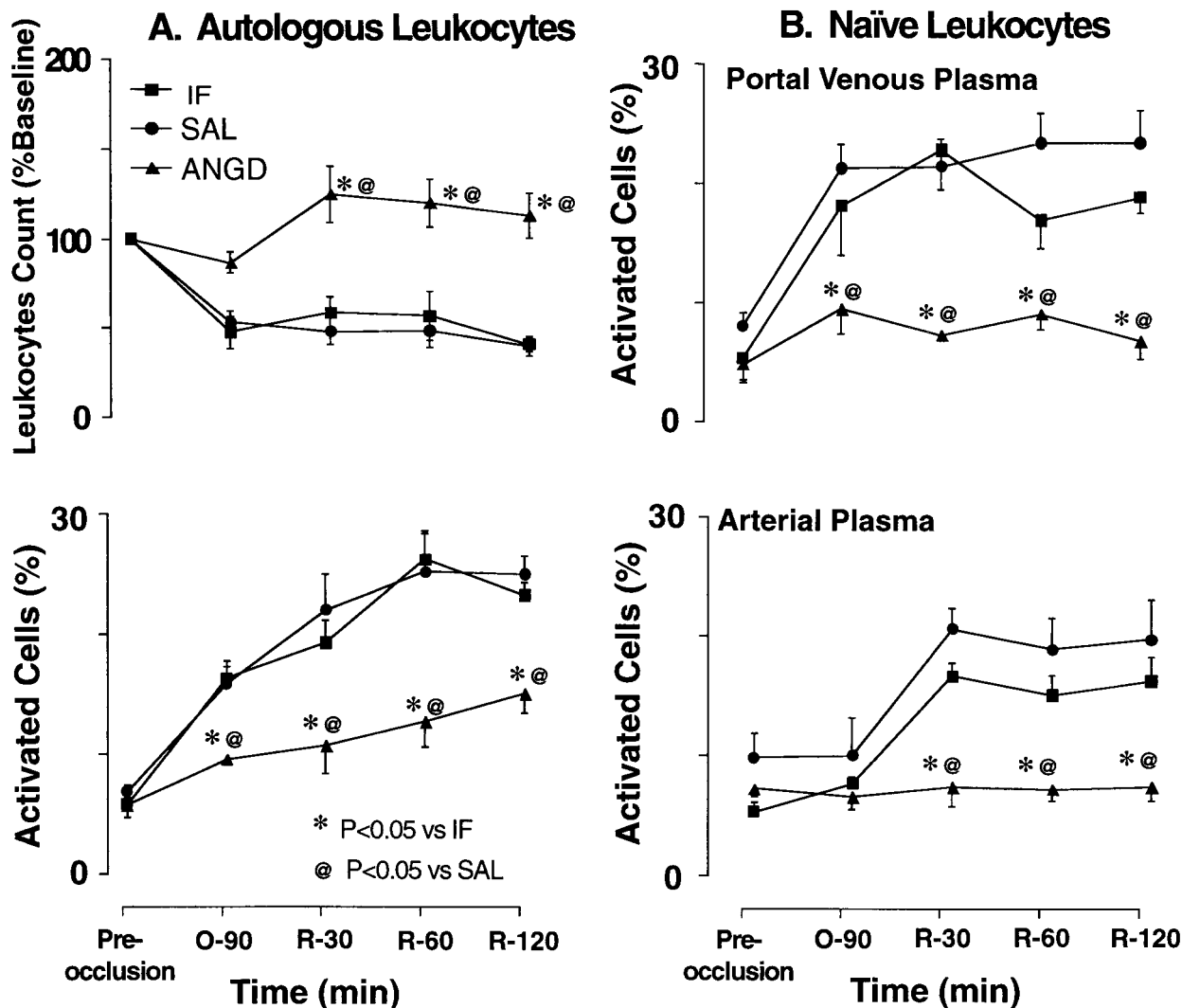


Fig. 2. (A) Activation of circulating leukocytes and leukocyte count. Numbers on the abscissa indicate the minutes after splanchnic arterial occlusion (O) and reperfusion (R). $P < 0.01$ at 30, 60, and 120 min of reperfusion. (B) Activation of naïve leukocytes induced by rat plasma in splanchnic arterial occlusion shock. Numbers on the abscissa indicate the minutes after splanchnic arterial occlusion (O) and reperfusion (R). * and @, $P < 0.05$, vs. IF and SAL groups, respectively.

In vitro production of leukocyte activator. Without trypsin, a low level of activation was detected in intestinal homogenates incubated for 120 min at 37°C. Intestinal homogenates incubated with trypsin, however, increased the ability to activate naïve leukocytes in a time- and trypsin concentration-dependent manner (Fig. 4).

Discussion

The current study indicates that pancreatic digestive enzymes in the intestinal lumen may form *in vivo* activators during ischemia and reperfusion of the intestine. These *in vivo* activators lead to up-regulation of cells in the circulation and compromise of microcirculatory functions. A key issue is the localization of pancreatic digestive enzymes. The pancreas serves as a source for digestive enzymes, and if homogenized under *in vitro* conditions, yields a large amount of potent activators (10). But as shown here, occlusion of the pancreatic blood supply during splanchnic arterial occlusion has no significant impact on cell activation or multiple organ failure. Instead, as part of normal digestion, the pancreas discharges into the intestine. In the presence of pancreatic enzymes, the ischemic intestine produces powerful *in vivo*

activators whose effect can be detected relatively early during ischemia in portal venous and central plasma. With release of the activators, the initial signs of multi-organ failure become apparent. These include arterial pressure reduction, leukopenia with infiltration of leukocytes into the microcirculation of the lung, liver, and other organs (19), pulmonary edema formation, morphological damage to the intestine, reduced liver function, and other cell and organ dysfunctions. The current results show that these processes are already attenuated by intestinal fluid lavage associated with reduced enzyme activity, and they are almost completely eliminated after protease blockade in the intestinal lumen.

Abdominal organs, such as intestine, have been the focus of shock research for several decades. Surgical excision of the small intestine increases survival of rats after hemorrhagic shock (20). But intestinal tissue by itself is not necessarily a source of a strong activator (Fig. 4). Our *in vitro* studies indicate that with the exception of the pancreas, homogenates of a variety of internal organs, including the intestine (after careful *in vitro* lavage), are less effective in activating leukocytes. Pancreatic enzymes or serine proteases *per se* (trypsin and chymotrypsin in saline after

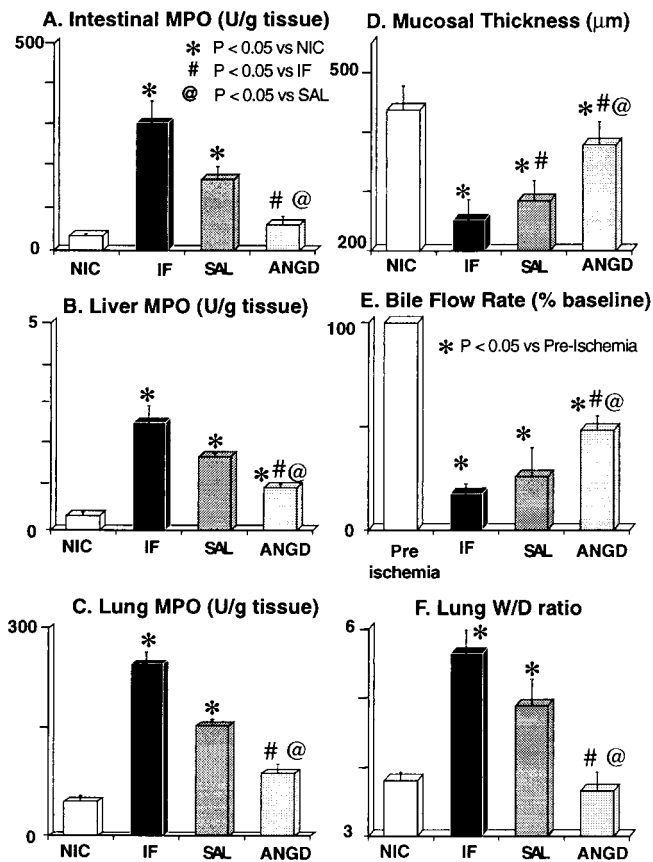


Fig. 3. (A–C) Tissue myeloperoxidase levels (MPO) in intestine (A), liver (B), and lung (C) after 90 min of reperfusion. (D) The level of intestinal injury as measured by mucosal thickness. (E) Bile flow rate as a measure of liver function. (F) Pulmonary dry/wet weight ratio. The abscissa indicates the time during splanchnic arterial occlusion (O) and reperfusion (R). NIC represents the nonischemic control. *, #, and @, $P < 0.05$, vs. NIC, IF, and SAL groups, respectively, A–D and F. *, $P < 0.05$ vs. preischemic control in E.

incubation periods of 2 hr) are also ineffective in generation of leukocyte activation (10). Instead, incubation of tissue from different organs, including the intestine, with pancreatic proteases for 2 hr leads to the production of humoral activators. These activators have an impact on a variety of cell functions, including production of oxygen free radicals in endothelial cells and leukocytes, expression of leukocyte adhesion molecules, and initiation of the leukocyte adhesion cascade to the point of parenchymal cell apoptosis (10). The activators, derived from intestine incubated with pancreatic enzymes, may have different molecular masses and include materials <2 kDa (10). Such small products may serve as humoral activators by entry into the systemic circulation through the portal vein and the lymphatics (1, 12, 21), especially if the permeability in an ischemic and damaged intestine is increased (11, 22). Proteases (23) as well as oxygen free radicals (24) may be involved in production of morphological damage to the intestinal villi. Perfusion of the intestinal lumen with a combination of ANGD and allopurinol (10 mg/ml) to block xanthine oxidase during splanchnic artery occlusion and reperfusion does not lead to further improvement of blood pressure (Fig. 1), reduction of activators (Fig. 2), or organ function (Fig. 3) compared with ANGD alone, and ANGD does not inhibit the conversion of xanthine dehydrogenase into xanthine oxidase (results not shown).

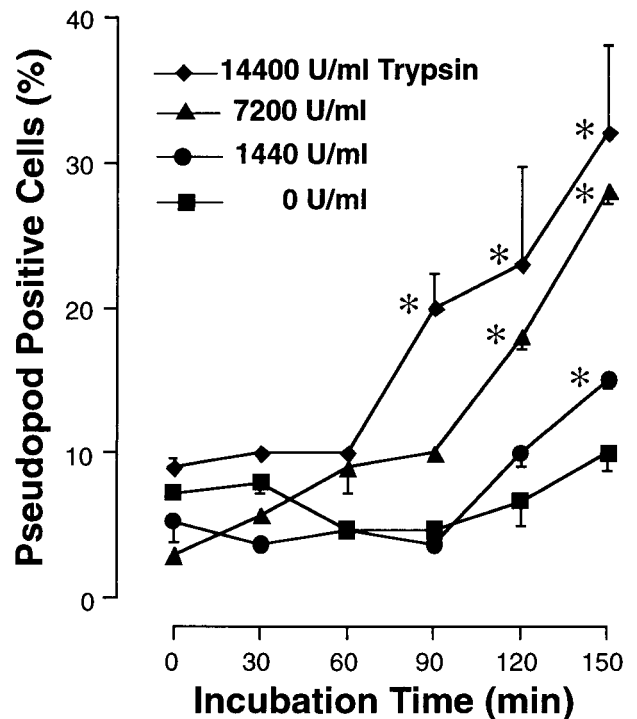


Fig. 4. Activation of naive leukocytes by intestinal homogenates incubated with increasing concentrations of trypsin. *, $P < 0.05$ vs. the values at 0 min.

Translocation of bacteria and endotoxins from the intestinal lumen into the lymphatics and vasculature has been proposed as a trigger mechanism for multiple-organ failure (25, 26). Endotoxin may induce shock, but there is no conclusive evidence that endotoxin translocation under experimental conditions serves as the main mechanism for inflammation (27, 28). The current evidence suggests that in addition to endotoxins, there are alternative mediators for cell activation. In light of the evidence that trypsin increases the ability of intestinal homogenates to activate, it is possible that activators are produced in the ischemic intestine by mechanisms that are influenced by protease activity in the lumen. A high protease activity in the ischemic intestine was detected even after intestinal lavage with saline (SAL group). In addition to an incomplete washout of pancreatic enzymes from the high number of minute crevices among intestinal villi, possible sources may be the conversion of trypsinogen, not removed by intestinal lavage (23), by enterokinase and/or lysosomal proteases from ischemic tissue (29, 30). Other sources may involve the complement or coagulation system, in which trypsin-like serine proteases are involved (31), and intestinal mast cells or activated leukocytes. Enzymes, which can be activated or released by proteolytic reactions, may be involved in the production of activators. For example, phospholipase A_2 may be an important enzyme in the production of stimulatory lysophospholipids, such as platelet-activating factor (32). Zymogen of pancreatic phospholipase A_2 is activated by trypsin. Inflammation caused by proteases or their products may also stimulate the secretion of phospholipase A_2 from the intestine.

The choice of enzyme inhibitor in the current study was governed by the fact that among five different inhibitors (Complete, phenylmethylsulfonyl fluoride, benzamidine, and aprotinin), ANGD was the most effective blocker for production of activators *in vitro* in the presence of pancreatic enzymes (10), providing $\approx 80\%$ reduction of activation. ANGD is a broad-

acting serine protease inhibitor (IC_{50} for trypsin = 1.3×10^{-8} M; IC_{50} for chymotrypsin = 5.9×10^{-4} M), which, at the concentrations used in the current experiments (0.37 mM) also blocks lipase ($IC_{50} = 8.4 \times 10^{-6}$ M) and phospholipase A_2 ($IC_{50} = 7.5 \times 10^{-5}$ M) (14) but has less effect on elastase or other enzymes. ANGD (at concentrations between 10^{-13} M and 10^{-3} M) does not significantly deactivate naive leukocytes after stimulation with plasma of rats after splanchnic arterial occlusion (collected at 30 min after reperfusion) or after stimulation with fMet-Leu-Phe (at 10^{-8} and 10^{-9} M; results not shown). Future use of enzyme blocker may involve even broader spectra to prevent activator production. Identification of the particular species constituting the plasma activator is the subject of current research (33).

The interaction between activated leukocytes and endothelial cells leads to accumulation of leukocytes in various organs, cytotoxicity, and cell death (34). Although such a process is mediated by humoral activators in the plasma of the systemic circulation, an inflammatory reaction is initiated in innocent-bystander organs that eventually may lead to multi-organ failure (35). When leukocytes are activated, the intracellular F-actin formation with pseudopod formation is up-regulated and several

membrane adhesion molecules are expressed, a process that lowers cell deformability and leads to accumulation of leukocytes in the microcirculation (36). Not only may such leukocytes start inflammation but also the abnormal cellular entrapment in the microcirculation leads to immune suppression because of reduced numbers of circulating cells. Suppression of plasma activator production serves to maintain normal leukocyte counts in the circulation (Fig. 2). Furthermore, the plasma-derived activators may also have direct suppressive effects on the parenchymal cell function without the requirement for leukocyte involvement (10).

In summary, we provide evidence that the small intestine in splanchnic arterial occlusion shock plays a role in activation of circulating blood cells by a mechanism that is controlled by pancreatic enzyme activity. Activators can be produced in the ischemic intestine and then enter the systemic circulation, impair multiple cell functions, and trigger a cascade that results in multi-organ failure. The effect can be blocked by inhibitors of pancreatic enzymes in the lumen of the intestine, a procedure that may have clinical application.

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