Clinical and experimental studies on treatment of acute mesenteric ischemia
Schoots, I.G.

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Inhibition of coagulation and inflammation by activated protein C or antithrombin reduces intestinal ischemia/reperfusion injury in rats

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Anticoagulant therapeutics – anti-inflammatory benefits

Abstract

Objective: To examine whether administration of activated protein C or antithrombin reduces local splanchnic derangement of coagulation and inflammation and attenuates intestinal dysfunction and injury following intestinal ischemia/reperfusion.

Design: Randomized prospective animal study.

Setting: University research institute.

Subjects: Adult male Wistar rats, weighing 300-325 g (n = 72).

Interventions: Rats were subjected to superior mesenteric artery occlusion consisting of 20 or 40 minutes ischemia and 3 hours of reperfusion. A randomized intravenous administration of vehicle (0.9% NaCl), heparin, antithrombin, or activated protein C was performed during ischemia, 15 minutes before reperfusion. Coagulation and fibrinolysis parameters obtained from portal blood, were correlated to mucosal fibrin deposition (determined by anti-rat fibrin antibody staining), intestinal function (glucose/water clearance) and intestinal injury (histological evaluation by Park/Chiu score).

Measurements and Main Results. Activated protein C or antithrombin treated animals demonstrated less ischemia/reperfusion-induced intestinal dysfunction and histological changes compared to control animals, whereas intravenous administration of heparin only showed less histological derangement. Activated protein C or antithrombin treated animals showed less thrombin generation, fibrin degradation products and fibrin deposition compared to control animals, as confirmed by histological examination, whereas heparin administration showed only a limited reduction of portal fibrin degradation products levels. Furthermore, activated protein C or antithrombin administration markedly inhibited the inflammatory response, as reflected by reduced interleukin-6 plasma levels to baseline values whereas heparin had no effect.

Conclusions: Administration of activated protein C or antithrombin inhibited local and systemic derangement of coagulation and inflammation following intestinal ischemia/reperfusion, diminished mucosal fibrin deposition and attenuated ischemia/reperfusion induced intestinal injury. These observations suggest that activated protein C or antithrombin reduces ischemia/reperfusion-induced intestinal injury, both through their anticoagulant and anti-inflammatory effects.
Introduction

The clinical course of critically ill patients can be complicated by the development of intestinal ischemia. The mesenteric hemodynamic response to severe sepsis and septic shock diminishes mesenteric blood flow and hence oxygen delivery in combination with an increased metabolic demand and enhances the ischemic state of the gut. Ischemia-reperfusion (I/R)-induced endothelial cell injury results in a procoagulant and fibrinolysis-suppressing environment giving rise to intra- and extravascular fibrin deposition which will further compromise the (micro)circulation of the intestine and promote necrosis in distal tissue. Mechanisms that have been incriminated to play a role in the procoagulant response are the upregulation of tissue factor in combination with dysfunctional anticoagulant pathways, along with suppression of fibrinolysis mainly due to increased levels of the inhibitor of fibrinolysis: plasminogen activator inhibitor (PAI)-1.

Regulatory anticoagulant pathways, in particular the antithrombin system and the protein C system, appear to be ineffective in inhibiting thrombin generation following I/R. Physiological anticoagulants such as antithrombin (AT) and activated protein C (APC), in addition to reducing thrombin generation, may exert anti-inflammatory properties including modulation of cytokine expression, regulation of cell migration and promotion of apoptosis. Restoration of these defective, physiological anticoagulant mechanisms form a logical approach to the (supportive) treatment of local or remote post-ischemic reperfusion injury.

AT, as a serine protease inhibitor, binds to glycosaminoglycans expressed on the endothelium at the site of the reperfusion injury and inhibits directly the proteolytic activity of generated thrombin. AT and other activated proteases have potent anti-adhesive and anti-inflammatory properties and inhibit the P-selectin-dependent leukocyte rolling and subsequent recruitment of leucocytes into tissues affected by I/R. Administration of AT has been shown to reduce liver and renal I/R injury by reversing this endothelial-leukocyte response to thrombin.

Protein C, as a circulating inactive vitamin K-dependent plasma glycoprotein, is converted to its active form by the endothelial surface-associated thrombin-thrombomodulin complex. APC and its cofactor protein S inactivate the coagulant factors Va and VIIIa. In addition, APC has also potent profibrinolytic and anti-inflammatory properties. APC administration has been shown to reduce cerebral infarct size and brain edema, to suppress endothelial ICAM-1 and to reduce myeloperoxidase formation in a murine model of focal cerebral ischemia. Previous studies have shown that APC inhibits leukocyte activation and cytokine-induced neutrophil chemoattractant expression following I/R in rat kidney, spinal cord and liver.

Owing to the anticoagulant and anti-inflammatory functions described above, AT and APC have a potential as prophylactic or therapeutic agents in the prevention or inhibition of microvascular dysfunction, inflammatory response and reperfusion injury following intestinal I/R. The present study was undertaken to investigate the anticoagulant and anti-inflammatory effects of anticoagulants, including unfractionated heparin, AT and APC, on the derangement of coagulation and inflammation in the splanchnic and systemic circulation following mild and moderate intestinal I/R in rats.
AT or APC inhibits intestinal ischemia/reperfusion injury

Materials and Methods

Biological and chemical agents
Plasma derived AT concentrate was obtained from Baxter (Vienna, Austria), recombinant-human APC concentrate was provided by Eli Lilly and Company (Indianapolis, IN), and unfractionated heparin was from Leo Pharma B.V. (Ballerup, Denmark). Goat anti-rat fibrin polyclonal antibody was kindly provided by Dr. J.J. Emeis (TNO Prevention and Health, Leiden, the Netherlands) (25). All other reagents were of analytical grade.

Animal model of intestinal I/R
Adult male Wistar rats (Charles Rivers, Broekman Instituut BV, Someren, the Netherlands), weighing 300-325 g, were fed standard rat chow (Hope Farms, Woerden, the Netherlands) and water ad libitum. The rats were allowed to acclimatize to our laboratory conditions for at least 4 days and were subjected to a regimen of 12:12 h / day-night cycle in mesh stainless-steel cages at constant temperature (22°C). The protocol was approved by the Animal Ethics Committee of the University of Amsterdam (the Netherlands). All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care and handling of laboratory animals. The last 12 hours prior to the experiments, the animals had no access to solid food, but free access to water.

Animal model used has been described previously 6. Briefly, under anaesthesia (1-2% isoflurane) and continuous monitoring of mean arterial pressure (90-110 mmHg) and body temperature (37±0.5°C), an intestinal loop of approximately 15 cm (10 cm proximal from the cecum) was isolated and canulated with soft silicon tubes, and connected to a perfusion pump, a heat exchanger and a reservoir to obtain a closed circuit. The reservoir contained freshly made Ringer’s solution consisting of glucose (25 mM), in order to evaluate the absorptive function of the intestinal epithelium.

Intestinal ischemia was induced by temporarily occlusion of the superior mesenteric artery (SMA) and confirmed by immediate blanching of the small intestine and cecum. Restoration of blood flow to the gut after declamping of the SMA was confirmed by returning to its original color. During reperfusion, luminal perfusion (1.0 mL/min) of the isolated intestinal loop was performed with the Ringer-glucose solution and samples from the perfusate (0.25 mL each) were obtained after 1, 2 and 3 hours of reperfusion and stored at -20°C until further analysis. Portal blood samples (1.25 mL each) were collected in sodium-citrate buffer (final citrate concentration 0.32%) and in EDTA tubes after 5 minutes, and after 1 and 3 hours of reperfusion, were centrifuged at 2000g at 4°C for 20 minutes and stored at -80°C until further analysis. Rats were sacrificed by bleeding after final blood sampling and subsequent administration of heparin (2 000 IU/kg) to prevent intravascular clotting. Biopsies of the small intestine were collected 5 cm proximal from the isolated rat intestinal loop and were fixed in 10% formaldehyde for histological examination.

Experimental design of intestinal I/R
In total, 72 rats were randomly allocated to a control group (saline) and three experimental groups (heparin, AT and APC, respectively), consisting of 18 rats each. Saline (0.9% NaCl), heparin (375 U/kg of body weight), AT (250 U/kg) or APC (100 μg/kg) was administered intravenously into the penile vein during the ischemic period, 15 minutes before reperfusion.

There is no single test that can directly match the anticoagulant effect of heparin, antithrombin and activated protein C. Therefore, we have chosen doses of each of these three agents that will result in plasma levels that are comparable to those achieved in clinical practice and have been shown to possess therapeutic antithrombotic potential in previous studies 15,16,20,22,26,27. Therapeutic doses of heparin in clinical studies result in anti-factor Xa levels of 0.5-1.0 IU/ml. Previous studies from our group have shown that a bolus subcutaneous injection of 375 IU/kg in a rat results in such plasma levels 28 and therefore this dose was chosen. Recent trials with antithrombin concentrate used dosages of approximately 250 to 300 IU/kg. Studies with 250 IU/kg
antithrombin concentrate have shown efficacy in animal models of endotoxemia and ischemia/reperfusion, whereas 50 or 100 IU/kg did not. The therapeutic dose of recombinant activated protein C in clinical studies is 24 μg/kg/hr. For technical reasons, we have chosen to administer activated protein C as a bolus and in view of the half-life of this agent a dose of 100 μg/kg was selected. In other studies the administration of this dose has demonstrated attenuation of liver and renal ischemia/reperfusion injury in rats. Based on the pharmacokinetics of this dose, therapeutic plasma levels during 2-4 hours of the experiment can be expected.

Each bolus injection of 1 mL/kg vehicle or anticoagulant was administered within seconds. Before reperfusion was induced by declamping the superior mesenteric artery, the anticoagulant equilibrated in the systemic circulation during 15 minutes, affecting the groups of 20 and 40 minutes of intestinal ischemia equally.

In each group, intestinal ischemia and reperfusion was induced by isolating and clamping the SMA with anatraumatic clamp for 0 minutes (sham operation, n = 6), 20 minutes (n = 6) or 40 minutes (n = 6), followed by 3 hours of reperfusion.

**Histological assessment of intestinal injury**

Histological grading of intestinal injury of formaldehyde fixed jejunal tissues, counterstained with haematoxylin and eosin, was performed by two independent, non-informed examiners, using the Park-Chiu classification.

**Assessment of intestinal transport**

Intestinal water transport (Cl\textsubscript{water}) was assumed to be reflected by the clearance of water from the total volume of the perfusion solution in the closed circuit (including the reservoir, connecting tubes and isolated intestinal loop) and was used to estimate net intestinal absorption and secretion. Glucose transport (Cl\textsubscript{glucose}) was determined to measure the active-transport capacity of the epithelium. The glucose concentration (C\textsubscript{Glucose}) was determined by a ‘Glucose Assay Reagent’ utilizing the hexokinase-glucose 6-phosphate dehydrogenase enzymatic assay (Sigma Diagnostics, St. Louis, MO, USA). The transport rate of water and glucose was determined as the clearance from the luminal perfusate per minute per gram intestine and calculated from the formula:

\[
\text{Clearance (in } \mu \text{L/g.min)} = (C_i \times V_i - C_f \times V_f) / (0.5 \times (C_i + C_f) \times T \times W)
\]

in which C is the detectable glucose concentration of the initial solution (i) and final solution (f), V the volume of the same solutions, T the time in minutes (min), and W the weight of the intestinal loop in gram (g).

**Assessment of coagulation and fibrinolysis**

Plasma samples in sodium-citrate buffer, stored at -80 °C were utilized. Thrombin generation was assessed by measuring the thrombin-antithrombin (TAT) complexes with an enzyme-linked immunosorbent assay (ELISA) kit (Behring, Marburg, Germany). AT was measured by an automated amidolytic technique according to methods previously described. Fibrin degradation products (D-dimers) were obtained by an ELISA (Asserachrom D-Di, Diagnostica Stago, Asnieres-sur-Seine, France). Plasminogen activator activity (PAA) and plasminogen activator inhibitor (PAI)-1 activity were measured by amidolytic assays previously described.

**Immunohistochemical assessment of fibrin deposition**

Fibrin deposition was detected on formaldehyde-fixed tissue sections using immunohistochemistry according to standard procedures, described previously. As negative controls, parallel sections consisted of the omission of the primary antibody and yielded no immunohistochemical reaction. Microscopical evaluation of fibrin deposition was performed by two blinded examiners.
**Measurement of cytokines**
Levels of rat tumor necrosis factor-α, cytokine induced neutrophil chemoattractant, interleukin-1β and interleukin-6 were determined with the use of rat ELISA kits (R&D Systems, Minneapolis, MN) in EDTA plasma samples, stored at -80 °C.

**Statistical analysis**
The data analysis was performed using Graphpad Prism version 3.0 (Graphpad Software, Inc) for Windows 95. Quantitative data were presented as median values and interquartiles or as mean values ± standard error of the mean (SEM). Differences between experimental groups for repeated measurements were analyzed by analysis of variance (ANOVA) and subsequent Bonferroni’s post-hoc test. Differences between experimental groups for single measurements were analyzed by the unpaired student-‘t test. Mann-Whitney U test was only used for analysis of the histology scores because equal variance and normal distribution conditions were violated. P values <0.05 were considered to be statistically significant.

**Results**

**Effects of heparin, AT and APC on morphology of I/R-induced intestinal injury**
Microscopical examination of intestinal tissue revealed subepithelial space in the villus tips with moderate to massive lifting, together with villus denudation in saline treated animals after 20 minutes of ischemia and 3 hours of reperfusion, showing a median score of 3.5 (range 2-4, P=0.007) (Figure 1). Intestinal injury was higher to the extent of disintegration of the lamina propria in most intestinal tissues of saline treated animals after 40 minutes of ischemia, yielding a median score of 5 (range 4-5, P=0.001); such changes were not observed in sham-operated animals. Intravenous administration of heparin, AT or APC demonstrated significantly less intestinal injury after 40 minutes of ischemia and 3 hours of reperfusion to median scores of 3 (heparin, \( P=0.021 \)), 4 (AT, \( P=0.046 \)) and 3.5 (APC, \( P=0.032 \)), respectively. There was a non-significant trend towards a lower score after administration of heparin, AT and APC after 20 minutes of SMA occlusion.

**Figure 1. Effects of heparin, antithrombin and activated protein C on morphology of I/R-induced intestinal injury**
Histological analysis of intestinal tissues was performed after 3 hours of reperfusion and assessed according to the Park-Chiu classification. Data of animals treated with saline (closed bars), heparin (hatched bars), antithrombin (AT) (crossed bars), or activated protein C (APC) (blocked bars) are presented as median values and interquartiles (n = 6, in each group). *\( P<0.05 \) compared with the sham-operated group; †\( P<0.05 \) compared with 20 minutes I/R plus saline group; ‡\( P<0.05 \) compared with 40 minutes I/R plus saline group.
**Effects of heparin, AT and APC on I/R-induced intestinal dysfunction**

Intestinal clearances of glucose and water during 3 hours of reperfusion were significantly and dose-dependently decreased after 20 and 40 minutes of intestinal ischemia in saline treated animals (Figure 2). Administration of heparin, AT or APC did not improve intestinal dysfunction after 40 minutes of ischemia and 3 hours of reperfusion; however, administration of AT or APC significantly attenuated intestinal dysfunction after 20 minutes of ischemia and 3 hours of reperfusion to baseline glucose and water clearance levels. This was in particular the case for glucose clearance. Heparin failed to show any improvement of intestinal dysfunction after 20 minutes of ischemia and 3 hours of reperfusion.

**Figure 2. Effects of heparin, antithrombin and activated protein C on I/R-induced intestinal dysfunction**

Intestinal clearances of glucose and water during 3 hours of reperfusion were determined using an intestinal loop of approximately 15 cm. Values represent the total clearance following 3 hours of reperfusion. Data of animals treated with saline (closed bars), heparin (hatched bars), antithrombin (AT) (crossed bars), or activated protein C (APC) (blocked bars) are expressed as mean values ± SEM (n = 6, in each group). *P<0.05 compared with the sham-operated group.

**Effects of heparin, AT and APC on intestinal I/R-induced activation of coagulation and fibrinolysis**

Portal plasma levels of the coagulation parameters TAT, AT, FDP (D-dimer), PAA and PAI-1 in the sham-operated animals were in the normal range and did not change during the whole experiment.

Intestinal ischemia and reperfusion resulted in local activation of coagulation. Thrombin generation and conversion of fibrinogen to fibrin occurred as reflected by
AT or APC inhibits intestinal ischemia/reperfusion injury

**A**

TAT (ng/mL) vs. Time of reperfusion (min)

**B**

TAT (ng/mL) vs. Time of reperfusion (min)

**C**

ATII (%) vs. Time of reperfusion (min)

**D**

ATII (%) vs. Time of reperfusion (min)

**E**

FDP (ng/mL) vs. Time of reperfusion (min)

**F**

FDP (ng/mL) vs. Time of reperfusion (min)
Figure 3. Effects of heparin, antithrombin and activated protein C on intestinal I/R-induced activation of coagulation and suppression of fibrinolysis

Activation of coagulation and suppression of fibrinolysis were determined by the measurement of portal plasma levels of thrombin-antithrombin (TAT)-complexes (A,B), antithrombin (AT) (C,D), fibrin degradation products (FDP) (E,F), plasminogen activator activity (PAA) (G,H) and plasminogen activator inhibitor (PAI)-1 (I,J). Animals were intravenously administered 0.9% saline, heparin, antithrombin (AT), or activated protein C (APC), 15 minutes before reperfusion. Data (n = 6, in each group) are expressed as mean values ± SEM. Portal plasma levels of repeated measurements during 3 hours of reperfusion in saline treated animals subjected to sham operation ( ), or to 20 minutes ( ) or 40 minutes ( ) of ischemia are depicted in A, C, E, G and I. Portal plasma levels of animals treated with saline (closed bars), heparin (hatched bars), antithrombin (AT) (crossed bars), or activated protein C (APC) (blocked bars), subjected to 20 minutes or 40 minutes of ischemia after 3 hours of reperfusion are depicted in B, D, F, H and J. Values shown represent the 3 hour reperfusion time-point. *P<0.05 compared with the sham-operated group; †P<0.05 compared with 20 minutes I/R plus saline group; ‡P<0.05 compared with 40 minutes I/R plus saline group.
AT or APC inhibits intestinal ischemia/reperfusion injury

Figure 4. Effects of heparin, antithrombin and activated protein C on intestinal I/R-induced mucosal fibrin deposition and microvascular thrombosis

Representative results of mucosal fibrin deposition after 3 hours of reperfusion are shown of animals in the sham-operated group (A and B), and of animals subjected to 40 minutes of ischemia, treated with saline (C and D), heparin (E and F), antithrombin (G and H), or activated protein C (I and J). For A, C, E, G and I, original magnification x200 (bar in A represents 50 µm); for B, D, F, H and J, original magnification x500 (bar in B represents 20 µm). Location of enlargement (asterisk (*)), lumen (L), villus (V), and epithelium (E) are indicated. Arrows indicate fibrin deposition (brown-stained areas).
increase of the portal plasma levels of TAT-complexes and FDP (Figure 3). During 3 hours of reperfusion portal plasma levels of TAT-complexes increased 3-fold and 5-fold after 20 and 40 minutes of ischemia, respectively. Generation of thrombin and formation of TAT-complexes resulted in local consumption of AT to $92\pm1\%$ ($P<0.001$) and to $86\pm1\%$ ($P<0.001$) of baseline values after 20 and 40 minutes of ischemia, respectively.

Administration of AT demonstrated significant lower mesenteric thrombin generation after mesenteric I/R, as reflected by a 2-fold and 1.5-fold reduction of portal plasma levels of TAT-complexes and FDP, respectively, after 40 minutes of ischemia and 3 hours of reperfusion. Similar results were obtained after administration of APC. Administration of heparin resulted in a relatively small reduction of FDP levels ($P=0.049$) after 40 minutes of ischemia and 3 hours of reperfusion, whereas thrombin generation was not reduced, as reflected by comparable levels of TAT-complexes and AT values in portal plasma compared to plasma levels in saline treated animals.

After initial local activation of fibrinolysis after 1 hour of reperfusion, as demonstrated by an increase in portal PAA, fibrinolytic activity was subsequently depressed to $91\pm2\%$ ($P<0.002$) and to $81\pm2\%$ ($P<0.001$) of baseline levels 3 hours after 20 and 40 minutes of ischemia, respectively. This decrease of plasminogen activating activity was associated with an increase in portal plasma levels of PAI-1, reaching levels of $17\pm1$ IU/mL ($P<0.001$) and $25\pm2$ IU/mL ($P<0.001$) after 20 and 40 minutes ischemia and 3 hours of reperfusion, respectively.
Administration of APC significantly depressed plasma levels of PAI-1 after ischemia and reperfusion, which resulted in increased fibrinolytic activity. Neither heparin nor AT administration showed reduced PAI-1 levels, although heparin administration demonstrated a small increase in fibrinolytic activity after 40 minutes of mesenteric ischemia and 3 hours of reperfusion.

**Effects of heparin, AT and APC on intestinal I/R-induced fibrin deposition and microvascular thrombosis**

Microscopical assessment of intestinal tissues of saline treated animals revealed mucosal fibrin deposits and microvascular thrombotic obstructions after 20 (data not shown) and 40 minutes of intestinal ischemia and 3 hours of reperfusion (Figure 4C and 4D), whereas histological examination after sham operation did not reveal mucosal fibrin deposits (Figure 4A and 4B). Either AT or APC administration showed markedly less mucosal fibrin deposits (Figure 4G-J) after 40 minutes of ischemia and 3 hours of reperfusion, compared to saline administration.

**Effects of heparin, AT and APC on intestinal I/R-induced inflammation**

Portal plasma levels of tumor necrosis factor-α, cytokine induced neutrophil chemoattractant and interleukin-1β were below the 15 pg/mL detection limit. Portal plasma concentrations of interleukin-6 were significantly higher after reperfusion in rats subjected to 20 (P=0.046) and 40 (P=0.002) minutes of intestinal ischemia than after

![Figure 5. Effects of heparin, antithrombin and activated protein C on intestinal I/R-induced inflammation](chart)

The local pro-inflammatory response to intestinal I/R was determined by the detection of interleukin-6 in portal plasma. Data (n = 6, in each group) are expressed as mean values ± SEM. Interleukin-6 portal plasma levels of saline treated, sham operated animals ( ), or subjected to 20 minutes ( ) or 40 minutes ( ) of ischemia during 3 hours of reperfusion are depicted in A. Interleukin-6 portal plasma levels of animals treated with saline (closed bars), heparin (hatched bars), antithrombin (AT) (crossed bars), or activated protein C (APC) (blocked bars), subjected to 20 minutes or 40 minutes of ischemia after 3 hours of reperfusion are depicted in B. Values shown represent the 3 hour reperfusion time-point. *P<0.05 compared with the sham-operated group; †P<0.05 compared with 20 minutes I/R plus saline group; ‡P<0.05 compared with 40 minutes I/R plus saline group.
reperfusion in sham-operated rats (Figure 5). The increases of portal plasma interleukin-6 were significantly inhibited by either AT or APC administration, but not by the administration of heparin after 40 minutes of intestinal ischemia and 3 hours of reperfusion.

**Effects of heparin, AT and APC on intestinal I/R-induced splanchnic and systemic coagulation, fibrinolysis and inflammation**

In the systemic circulation, the markers of coagulation, fibrinolysis and inflammation were much less affected by intestinal I/R than those in the splanchnic (portal) circulation in the saline treated animals (Table 1); however, the systemic changes in coagulation, fibrinolysis, and inflammation mimicked the local splanchnic response. The markers measured in the systemic circulation did not evidence any systemic derangement of coagulation and inflammation after either AT or APC administration in rats subjected to 40 minutes of ischemia and 3 hours of reperfusion as baseline values were detected.

**Table 1. Effects of heparin, antithrombin or activated protein C on splanchnic and systemic plasma levels of parameters for coagulation, fibrinolysis and inflammation.**

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Coagulation</th>
<th>Systemic</th>
<th>Sham</th>
<th>I/R+ NaCl</th>
<th>I/R+ Heparin</th>
<th>I/R+ AT</th>
<th>I/R+ APC</th>
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<tbody>
<tr>
<td><strong>TAT (ng/mL)</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Splanchnic</td>
<td>5±1</td>
<td>25±2</td>
<td>27±1</td>
<td>13±1</td>
<td>10±0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic</td>
<td>5±1</td>
<td>10±1</td>
<td>9±1</td>
<td>6±1</td>
<td>7±1</td>
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<td></td>
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<tr>
<td><strong>AT (IU/mL)</strong></td>
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<tr>
<td>Splanchnic</td>
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<td>85±1</td>
<td>84±1</td>
<td>340±10</td>
<td>94±1</td>
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<tr>
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<td>92±1</td>
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<tr>
<td><strong>FDP (ng/mL)</strong></td>
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<td>196±13</td>
<td>142±9</td>
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<td>101±3</td>
<td>84±4</td>
<td>88±3</td>
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<td><strong>Fibrinolysis (%)</strong></td>
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<tr>
<td>Splanchnic</td>
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<td>81±2</td>
<td>94±3</td>
<td>85±4</td>
<td>105±2</td>
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<tr>
<td>Systemic</td>
<td>101±1</td>
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<td>98±1</td>
<td>100±2</td>
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<td><strong>PAI-1 (IU/mL)</strong></td>
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<tr>
<td>Splanchnic</td>
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<td>25±2</td>
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<td>29±2</td>
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<tr>
<td><strong>Inflammation</strong></td>
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<tr>
<td>IL-6 (pg/mL)</td>
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<tr>
<td>Splanchnic</td>
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<td>60±6</td>
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<td>148±29</td>
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</table>

TAT, trombin-antithrombin complex; AT, antithrombin; FDP, fibrin degradation products; PAA, plasminogen activator activity; PAI-1, plasminogen activator inhibitor-1; IL-6, interleukin-6. Values shown represent the 3 hour reperfusion time-point. \*P<0.05 compared with systemic plasma levels of the same group. \^P<0.05 compared with the systemic plasma levels of sham-operated group.
AT or APC inhibits intestinal ischemia/reperfusion injury

Discussion

Splanchnic ischemia and gut mucosal injury have been incriminated to play a role in the development and maintenance of the systemic inflammatory response, a key factor causing multiple organ failure. APC and AT exert anticoagulant and anti-inflammatory properties and may therefore be utilized as prophylactic or therapeutic agents in the prevention or inhibition of microvascular dysfunction and inflammatory response following intestinal I/R.

In saline treated animals, 20 or 40 minutes occlusion of the SMA induced a procoagulation diathesis in the intestinal microcirculation during reperfusion. Marked thrombin generation, as evidenced by elevated thrombin-antithrombin complex levels and consumption of AT resulted in fibrinogen-to-fibrin conversion, indicated by high levels of FDP (D-dimer). In addition, fibrin deposits seemed to be inadequately removed, due to a dysfunctional fibrinolytic system caused by high levels of PAI-1. This inhibition of fibrinolysis was preceded by a short-lasting increase in PAA, most probably caused by immediate release of plasminogen activator by endothelial cells upon injury. The resulting effect of fibrin generation and inadequate fibrin removal resulted in demonstrable intravascular fibrin deposition in the intestinal microcirculation following I/R.

Pharmacological doses of either APC or AT, both physiological anticoagulants significantly inhibited I/R-induced intestinal thrombin generation, fibrin formation and fibrin deposition after mild and moderate intestinal ischemia, whereas heparin showed only a limited reduction of plasma FDP levels. Restoration of the antithrombin system following AT administration reduced plasma levels of TAT-complexes, indicating reduced thrombin and fibrin generation. Interestingly, therapeutic administration of heparin was ineffective to decrease the coagulation activation following intestinal I/R, indicating that the endogenous amount of AT, available at the mesenteric endothelium, was not capable of inhibiting factors Xa and thrombin by heparin. Alternatively, it has been shown that the heparin-antithrombin complex is not able to block surface bound coagulation activation, which may also explain the failure of heparin to reduce coagulation activation. Considering combined treatment of heparin and antithrombin in this study, heparin may have resulted in adverse effects of heparin on the microcirculatory actions of antithrombin as shown during endotoxemia. It has been demonstrated that heparin competitively inhibits the binding of antithrombin to other glycosaminoglycans. Restoration of the protein C system following APC administration resulted in an equivalent decrease of TAT-complex formation and AT consumption, indicating increased inhibition of generated factor Xa by inactivation of factor Va and VIIIa, and attenuation of thrombin and fibrin generation after mild and moderate intestinal I/R. In addition, APC increased fibrinolytic activity as reflected by elevated plasminogen activator activity and reduced plasma levels of its inhibitor PAI-1. Both, AT and APC administration significantly diminished mucosal fibrin generation and deposition.

Fibrin deposition and microvascular thrombosis play a pivotal role in disseminated intravascular coagulation-associated multiple organ failure and I/R-induced injury, by compromising microcirculatory blood flow. I/R-induced intestinal dysfunction and histological changes after mild and moderate intestinal ischemia were less after either APC or AT administration compared to saline infusion, whereas heparin only reduced the histological sequelae of intestinal I/R. Heparin has been demonstrated to preserve intestinal perfusion and gut mucosal pO2 levels after hemorrhage and resuscitation in

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rats, which may account for the observed reduction in intestinal I/R injury. However, histological changes only reflect part of the damage caused by I/R and the ensuing coagulation and inflammatory activation. In fact, it has been shown that functional changes upon I/R do not always strongly correlate with structural changes. The attenuation of intestinal I/R injury following anticoagulant administration may result from increased microvascular perfusion owing to diminished fibrin deposits and microthrombotic obstructions, however, may also result from the anti-inflammatory properties mentioned above.

In support of the latter, the inflammatory response after intestinal I/R was lower following AT infusion compared to vehicle administration, as reflected by the reduction of interleukin-6 plasma levels to baseline values. The administration of APC also significantly inhibited the inflammatory response, whereas administration of heparin did not decrease post-ischemic interleukin-6. We have previously shown that systemic activation of coagulation is mainly driven by interleukin-6, whereas for changes in the anticoagulant and fibrinolytic pathways, tumor necrosis factor-α can be held responsible. Although in our model of mild to moderate intestinal I/R, local plasma levels of tumor necrosis factor-α, cytokine induced neutrophil chemoattractant and interleukin-1β were not detectable, previous studies have shown that tumor necrosis factor-α and cytokine-induced neutrophil chemoattractant following 'severe' I/R were suppressed after APC or AT administration.

Interestingly, the local splanchnic changes in coagulation and fibrinolysis mimic the systemic response upon a generalized inflammatory state; in this situation tissue-factor-driven thrombin generation is also insufficiently contained by dysfunctional anticoagulant pathways and inadequately balanced by a suppressed fibrinolytic system. This leads to widespread systemic intravascular fibrin deposition, eventually resulting in disseminated intravascular coagulation and subsequent organ failure in critically ill patients. Either APC or AT administration has been shown to effectively alleviate the coagulation abnormalities in patients with disseminated intravascular coagulation. As a result of the protective properties of these anticoagulants to I/R injury, we may speculate that APC and AT are beneficial not only to reduce coagulation abnormalities, but also to counteract intestinal injury in patients with disseminated intravascular coagulation associated with shock or sepsis in critically ill patients. In our model, the systemic circulatory events concerning coagulation and fibrinolysis were much less influenced by intestinal ischemia and reperfusion than those in the local, splanchnic (portal) circulation. Systemic monitoring of coagulation parameters (D-dimer) may not reveal splanchnic coagulation abnormalities after mild intestinal I/R. However, after 40 minutes of intestinal ischemia, systemic circulatory changes in coagulation and fibrinolysis showed significant alterations corresponding to the changes found in the portal circulation upon I/R. These observations indicate that a systemic procoagulant state may indeed occur upon intestinal I/R, which may damage tissue at a site remote from the initial ischemic event. Acute respiratory failure is the most important sequel in this clinical scenario. The administration of APC or AT concentrates completely reversed the systemic changes of coagulation activation after intestinal I/R in the present study, and therefore, may also diminish remote activation of coagulation and inflammatory response.
Conclusion

Intestinal I/R resulted in considerable local and systemic derangement of the coagulation and inflammatory system, compromising mucosal and submucosal microcirculation by widespread microthrombosis and deposition of fibrin. Administration of the physiological anticoagulants APC or AT inhibited the derangement of coagulation and inflammation following intestinal I/R, diminished mucosal fibrin deposition and decreased histological changes of intestinal injury. Although the early stage of treatment initiated and the short time window of this experimental study do not reflect the broad treatment spectrum of intestinal ischemia in a large variety of clinical settings, these observations may still be relevant for analyzing pathways in the pathogenesis of I/R injury and for the potential treatment of critically ill patients with intestinal ischemia. The present results may support the initiation of future clinical studies to investigate the potential benefit of this treatment.

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