Clinical and experimental studies on treatment of acute mesenteric ischemia
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Local intravascular coagulation and fibrin deposition upon intestinal ischemia and reperfusion in rats

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\textit{Ischemia-induced microvascular thrombotic obstruction – pivotal role or epiphenomenon?}

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Abstract

Background: The aim of this study was to investigate intravascular coagulation and thrombotic obstruction in the splanchnic vasculature after intestinal ischemia in relation to epithelial integrity and function.

Methods: Intestinal ischemia was induced in rats by superior mesenteric artery (SMA) occlusion for 20 or 40 minutes. Intestinal injury was assessed by histological analysis, biochemical markers and functional studies. During reperfusion, portal and systemic blood samples were collected to analyse activation of coagulation and fibrinolysis.

Results: SMA occlusion resulted in mild to moderate intestinal injury. Twenty and forty minutes of ischemia and 3 hours of reperfusion resulted in local intestinal thrombin generation and conversion of fibrinogen to fibrin, reflected by 3- and 4-fold increases in thrombin-antithrombin complex levels and a 3-fold elevation of fibrin degradation products (D-dimer), respectively. During reperfusion, after a short-lasting, initial activation of local fibrinolysis, plasminogen activator activity was suppressed, as indicated by an almost 4-fold increase in portal plasma levels of the plasminogen activator inhibitor. D-dimer levels showed that activation of coagulation and depression of fibrinolysis resulted in fibrin formation, which was confirmed to be intravascular fibrin deposition by histological examination.

Conclusions: Intestinal ischemia and reperfusion results in local intravascular coagulation and fibrin deposition.
Introduction

Intestinal ischemia is a condition that leads to considerable morbidity and mortality and is frequently seen in critically ill patients who suffer from hypovolemic or septic shock. The endothelium of the splanchnic vasculature is believed to play a major role in the development of intestinal infarction. The endothelium, phenotypically a non-thrombogenic surface, is capable of balancing the pro- and anticoagulant mechanisms that prevent intravascular coagulation. However, ischemia can disturb the endothelial balance of a non-thrombogenic state into a prothrombotic state 1,2. Ischemia-associated fibrin deposition and thrombosis are speculated to result from ischemia-induced changes of the microvascular microenvironment, including diminished aerobic metabolism, accumulation of waste products and activated inflammatory response.

Fibrin deposition after ischemia and reperfusion injury has received relatively little attention, although it has recently been shown to contribute to microvascular obstructions in early focal cerebral ischemia and reperfusion in rats 3-5. It was also suggested to be involved in the “no reflow” phenomenon, the paradoxical condition in which no perfusion occurs in the microvasculature during macrovascular reperfusion of ischemic tissue. Fibrin deposition in the microvasculature of the intestine after occlusive intestinal ischemia 6 promoted platelet adhesion, which is likely to contribute to the manifestation of microvascular ischemia and reperfusion injury. Although fibrin deposition and microthrombi in the intestine after ischemia was already reported in the early 1970s 7, the postischemic response and the interaction of the coagulation and fibrinolytic systems in the splanchnic circulation after intestinal ischemia and reperfusion have not been reported so far.

To evaluate the role of intravascular coagulation in microvascular reperfusion injury after acute mesenteric vascular occlusion, we assessed intravascular coagulation, fibrinolysis and subsequent intravascular fibrin deposition and microvascular thrombotic obstructions in the splanchnic circulation after mild and moderate intestinal ischemia and subsequent reperfusion in rats.

Material and Methods

Animals

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.

In total, 24 rats were randomly allocated to one sham group and the two experimental groups. Intestinal ischemia and reperfusion was induced by isolating and clamping the superior mesenteric artery (SMA) with an atraumatic clamp for 0 minutes (sham operation, n = 7), 20 minutes (n = 8) or 40 minutes (n = 9), followed by 3 hours of reperfusion. During the sham operation the mesenteric artery was isolated without clamping, followed by 3 hours of sham reperfusion.
Intravascular coagulation and fibrin deposition

To exclude different influences of anaesthesia upon the outcome parameters, all rats were anaesthetized for approximately 4.5 hours, which includes the duration of the surgical procedure and the period of ischemia and reperfusion.

**Surgical procedure**

Under anaesthesia with 1-2% isoflurane, rats were intubated and ventilated. CO$_2$ levels were kept between 37 and 43 mmHg. Continuous 0.9% saline-glucose solution (5mM) was infused via the tail vein (10ml/hr/kg body weight) to correct for possible fluid loss during the experiment. A canula was inserted into the left carotid artery to measure the mean arterial pressure, which was kept between 90 and 110 mmHg during the experiment. Body temperature was maintained at 37 °C by use of a heating pad and lamp.

A 5.0 cm long midline laparotomy was performed and an intestinal loop of approximately 15 cm (10 cm proximal from the cecum) was isolated and canulated with soft silicon tubes. This loop was gently rinsed with saline prior to connection 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 (in mmol/L): NaCl, 117.5; KCl, 5.7; NaHCO$_3$, 25.0; MgSO$_4$, 1.2; Na$_2$PO$_4$, 1.2; CaCl$_2$, 2.5 (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Glucose (2.5*10$^{-2}$M) and disodiumfluorescein (Na$_2$Fl, Molecular Probes, Leiden, the Netherlands) (1.0*10$^{-5}$M) were added to this solution, in order to evaluate the absorptive and barrier function of the intestinal epithelium.

The SMA was identified after deflecting the intestinal loops to the right side of the abdomen. The SMA was temporarily occluded by an atraumatic clamp at the origin of the aorta, avoiding the accompanying lymphatic trunk. Immediate blanching of the small intestine and cecum confirmed that the blood supply to the intestinal segments had been shut off. The abdomen was then covered with a sterile moist gauze pad. After the period of ischemia, the clamp was removed from the SMA and restoration of blood flow to the gut was verified by returning to its original colour.

During reperfusion, luminal perfusion (1ml/min$^{-1}$) of the isolated intestinal loop was performed with the Ringer-glucose-Na$_2$Fl solution. 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 after 5 minutes, 1 and 3 hours of reperfusion. Three times of portal blood sampling during the experiment was chosen to limit blood extraction (<15% of total blood volume). An equivalent volume of a warmed volume expander of 6% polyhydroxyethyl-starch and 0.9% NaCl (eloHaes, Fresenius Kabi, the Netherlands) was injected through the tail vein to maintain blood volume. After 3 hours of reperfusion, a blood sample (1.25 ml) was also taken from the carotid artery. Blood samples were collected in Na-citrate buffer (final citrate concentration 0.32%) or in EDTA tubes and were centrifuged at 2.000 x g at 4 °C for 20 minutes and the plasma samples were stored at −20 °C until further analysis. At the end of each experiment, a blood-gas analysis was performed.

The rat was sacrificed by bleeding after final blood sampling. 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.

**Assessment of intestinal injury**

**Histological analysis:** The formaldehyde fixed jejunal tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E) for histological grading. The histological grading classification of Park-Chiu was used by an independent, non-informed pathologist to assess intestinal injury. Briefly, the scores used were 0: normal mucosa, 1: subepithelial space at villus tips, 2: extension of subepithelial space with moderate lifting, 3: massive lifting down sides the villi, some denuded villi, 4: denuded villi, dilated capillaries, 5: disintegration of the lamina propria, 6: crypt layer injury, 7: transmucosal infarction and 8: transmural infarction.

**Plasma parameters of intestinal injury:** In EDTA plasma samples, lactate dehydrogenase (LDH) and alpha glutathion-S-transferase (αGST) levels were determined to evaluate (intestinal) cell
leakage following ischemia and reperfusion injury. LDH levels were obtained with a cytotoxicity detection kit (Roche Diagnostics GmbH, Mannheim, Germany) and αGST levels by using an enzyme-linked immunoassay (ELISA) (Biotrin, Dublin, Ireland).

Assessment of intestinal transport and barrier function

Intestinal water transport (Cl\text{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\text{glucose}) was determined to measure the active-transport capacity of the epithelium. The glucose concentration (C_{\text{Glucose}}) was determined by a glucose-detection kit (Sigma Diagnostics, St. Louis, MO, USA).

Intestinal epithelial barrier function for small molecules was assumed to be reflected by the clearance of Na₂Fl (Cl\text{Na₂Fl}) from the total volume, and was used to measure the passive transport of small water-soluble substances from the intestinal lumen into the tissue. The concentration of Na₂Fl (C_{\text{Na₂Fl}}) was obtained by using a fluorescence reader (Cytoflour 4000, PerSeptive Biosystems, Framingham, MA, USA) at excitation and emission wave lengths of 485 and 530 nm, respectively; the transport rate was determined as the clearance from the luminal perfusate of the Na₂Fl probe per minute per g intestine and calculated from the formula:

\[
\text{Clearance (in } \mu\text{L/g.min) = } \frac{(C_iV_i-C_fV_f)}{0.5(C_i+C_f)T\cdot W}
\]

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

Assessment of coagulation and fibrinolysis

Coagulation and fibrinolysis parameters. Collected blood samples were centrifuged for 20 minutes at 2,000 x g and plasma was stored at -20°C until assayed. Thrombin generation was assessed by measuring the thrombin-antithrombin (TAT) complexes with an ELISA (Behring, Marburg, Germany). Antithrombin III (ATIII) was measured by an automated amidolytic technique according to methods described by ten Cate et al.\textsuperscript{9}. Fibrin degradation products (D-dimers) were obtained by an ELISA (Asserachrom D-Di, Diagnostica Stago, Asnieres-sur-Seine, France), described by Elms et al.\textsuperscript{10}. Plasminogen activator activity (PAA) was measured by an automated amidolytic assay, described by Verheijen et al.\textsuperscript{11}. Briefly, 25 μl of plasma was mixed with 0.1 M TrisHCl, pH 7.5, 0.1% (v/v) Tween-80, 0.3 mM S-2251 (Chromogenix, Mölndal, Sweden), 0.13 M plasminogen and 0.12 mg/ml cyanogen bromide-digested fibrinogen fragments of fibrinogen to a final volume of 250μl. The concentration of PAA under these conditions is proportional to the amount of plasmin formed, which can be spectrophotometrically detected by conversion of the chromogenic substrate. Plasminogen activator inhibitor (PAI) activity was measured with the amidolytic method described by Levi et al.\textsuperscript{12}. Briefly, plasma was incubated with a fixed excess of t-PA (40 IU/ml) for 10 minutes at room temperature. The residual t-PA activity was determined by incubation with 0.13 μM plasminogen (Chromogenix, Sweden), 0.12 mg/ml cyanogen bromide-digested fibrinogen fragments and 0.1 mM S-2251 (Chromogenix, Sweden). Under these circumstances, PAI activity in the sample is inversely proportional to the plasmin generated in the incubation mixture, and can be determined by the conversion of the chromogenic substrate.

Fibrin deposition. Immunohistochemical detection of fibrin was performed using a polyclonal biotinylated goat anti-mouse fibrinogen antibody (Accurate chemicals, Boston, USA). Sections of jejunal tissue were washed and bound primary antibodies were detected by successive incubations with streptavidin / horseradish peroxidase (Lab Vision, Fremont, CA) and diaminobenzidine tetrachloride (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). We performed negative controls with non-specific immunoglobulin for immunohistochemical detection of fibrin. Evaluation of microvascular thrombosis was performed by immunohistochemistry and histological examination.
Statistical analysis

The data analysis was performed using Graphpad Prism version 3.0 (Graphpad Software, Inc) for Windows 95. All quantitative data were presented as mean values ± standard error (SE). Statistical analysis was performed by analysis of variance and subsequent Bonferroni’s post test. Where appropriate, differences between groups were analyzed by the Mann-Whitney test. Differences within groups were obtained by statistical analysis with the Wilcoxon test. P values < 0.05 were considered to be statistically significant.

Results

Intestinal injury in response to ischemia-reperfusion

All 24 rats survived the experiment. After 20 and 40 minutes of ischemia and 3 hours of reperfusion, the values for pH, base excess and HCO₃⁻ concentration in the arterial blood, which reflect the metabolic state of the animals, were within the normal range and did not differ between the groups (results not shown).

Intestinal injury after ischemia and reperfusion, graded according to the Park-Chiu classification was apparent in the biopsies after 20 minutes of ischemia, and was increased after 40 minutes of ischemia, with median scores of 1 (range 0-1) and 2 (range 1-4), respectively. Control rats (sham operation) did not show any intestinal injury.

During reperfusion LDH and α-GST levels in portal plasma were not different after 20 minutes of ischemia, however these levels were increased after 40 minutes of ischemia compared to those in the control group (Table 1).

<table>
<thead>
<tr>
<th>Ischemia (min)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>0</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reperfusion (min)</td>
<td>5</td>
<td>500±20</td>
<td>515±45</td>
<td>828±149 *</td>
<td>12±5</td>
<td>49±7 *</td>
</tr>
<tr>
<td>60</td>
<td>434±40</td>
<td>498±62</td>
<td>760±90 *</td>
<td>12±5</td>
<td>41±7 *</td>
<td>62±19 ± *</td>
</tr>
<tr>
<td>180</td>
<td>376±37</td>
<td>417±42</td>
<td>666±140 *</td>
<td>21±8</td>
<td>37±10 *</td>
<td>77±234 *</td>
</tr>
</tbody>
</table>

Values are presented as means ± standard error. * p < 0.05 vs 0 minutes of ischemia, ± p < 0.05 vs 20 minutes of ischemia.

Intestinal transport in response to ischemia-reperfusion

Intestinal clearance of glucose and water is shown in Figure 1. During reperfusion, the isolated intestinal loop in the control rats showed a constant rate of glucose and water clearance. SMA occlusion of 20 minutes did not result in impaired intestinal absorption for glucose and water, whereas 40 minutes of intestinal ischemia resulted in a significantly decreased intestinal absorption.

The absorption capacity of the intestinal loop for glucose was markedly reduced after 40 minutes of vascular occlusion but did not further decrease within 3 hours of reperfusion (Figure 1).
Figure 1. Clearance of intestinal glucose and water (µL/min/g) during 3 hours of reperfusion after 0 minutes (white bars), 20 minutes (grey bars) and 40 minutes (black bars) of intestinal ischemia. Bars are presented as mean values ± SEM. * p < 0.05 vs. control group.

Figure 2. Portal plasma levels of thrombin-antithrombin (TAT) complexes, antithrombin III (ATIII), fibrin degradation products (FDP), plasminogen activator activity (PAA) and plasminogen activator inhibitor-1 (PAI-1) during reperfusion after 0 minutes (control group), 20 minutes and 40 minutes of intestinal ischemia. Data are presented as mean values ± SEM. * p < 0.05 vs. 20 and 40 minutes of intestinal ischemia, # p < 0.05 vs. 20 minutes of intestinal ischemia.
Intravascular coagulation and fibrin deposition

However, the net water clearance showed a gradual decrease during reperfusion, turning into negative values after 2 and 3 hours of reperfusion.

The epithelial permeability to Na$_2$Fl did not show any significant change after 20 or 40 minutes of SMA occlusion. The values for Na$_2$Fl clearance from lumen to tissue after 3 hours of reperfusion were 9.4±4.5 µl/g/min for the control group, 12.8±4.2 µl/g/min after 20 minutes of ischemia, and 12.5±3.7 µl/g/min after 40 minutes of ischemia.

Activation of coagulation and fibrinolysis in response to ischemia-reperfusion

Portal plasma levels of the coagulation parameters TAT, ATIII, FDP (D-dimer), PAA and PAI of the control rats were in the normal range and did not change during the experiment. These sham operated rats also did not show any activation of the coagulation cascade during the whole experiment (Figure 2).

Ischemia and reperfusion resulted in local intravascular coagulation activation. Thrombin generation and conversion of fibrinogen to fibrin occurred as reflected by increase of the portal plasma levels of TAT-complexes and FDP. Portal plasma levels of TAT-complexes increased 3-fold and 4-fold after 20 and 40 minutes of ischemia, respectively. Maximum levels of TAT-complexes were measured 3 hours after the onset of reperfusion and were 16±1 ng/ml ($p<0.001$) after 20 minutes of ischemia and 22±2 ng/ml ($p<0.001$) after 40 minutes ischemia (as compared with 4.9±0.8 ng/ml in the control group). Portal FDP (D-dimer) levels increased from 72±8 ng/ml to 155±7 ng/ml ($p<0.05$) after 20 minutes ischemia and from 62±5 ng/ml to 148±15 ng/ml ($p<0.01$) after 40 minutes ischemia and 3 hours of reperfusion. Generation of thrombin and formation of TAT-complexes resulted in a consumption of local ATIII levels to 81±4 % ($p<0.001$) and to 85±3 % ($p<0.001$) of baseline values after 20 and 40 minutes ischemia, respectively.

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 63±6 % ($p<0.001$) and to 74±7 % ($p<0.001$) of baseline levels 3 h after 20 and 40 minutes of ischemia, respectively. This shut-down of plasminogen activating
activity was associated with an increase in portal plasma levels of PAI, starting at 1 hours of reperfusion and reaching levels of 22±3 IU/ml \((p<0.001)\) and 21±2 IU/ml \((p<0.001)\) after 20 and 40 minutes ischemia and 3 hours of reperfusion, respectively. The reduction in fibrinolytic activity after 1 hour is further reflected by a decrease in fibrin degradation, as evidenced by a reduction in portal FDP levels between 1 and 3 hours, while thrombin generation continued \(\text{(Figure 2).}\)

The intestinal activation of coagulation and simultaneous depression of fibrinolysis after 20 and 40 minutes of ischemia resulted in intravascular and extravascular deposition of fibrin in the intestine after 3 hours of reperfusion \(\text{(Figure 3).}\)

In the systemic circulation the markers of coagulation and fibrinolysis were much less affected by intestinal ischemia and reperfusion than those in the local (portal) circulation \(\text{(Table 2).}\)

**Table 2. Local (splanchnic) and systemic (carotid) plasma levels of coagulation and fibrinolysis parameters at 3 hours of reperfusion after intestinal ischemia.**

<table>
<thead>
<tr>
<th></th>
<th>TAT (ng/mL)</th>
<th>ATIII (IU/mL)</th>
<th>FDP (ng/mL)</th>
<th>PAA (%)</th>
<th>PAI (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>local</td>
<td>syst</td>
<td>local</td>
<td>syst</td>
<td>local</td>
</tr>
<tr>
<td>Pre-ischem</td>
<td>4.8±0.9</td>
<td>100±1</td>
<td>48±4</td>
<td>100±0</td>
<td>5.9±0.6</td>
</tr>
<tr>
<td>Ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.4±0.8</td>
<td>3.7±0.4</td>
<td>99±1</td>
<td>99±1</td>
<td>54±7</td>
</tr>
<tr>
<td>20</td>
<td>15.6±0.7*</td>
<td>5.4±0.6*</td>
<td>80±3*</td>
<td>100±2</td>
<td>155±7*</td>
</tr>
<tr>
<td>40</td>
<td>21.6±2.4*</td>
<td>6.7±0.5*</td>
<td>84±3*</td>
<td>97±2</td>
<td>148±15*</td>
</tr>
</tbody>
</table>

TAT, thrombin-antithrombin complexes; ATIII, antithrombin III; FDP, fibrin degradation products; PAA, plasminogen activator activity; PAI, plasminogen activator inhibitor. Values are presented as means ± SEM. Pre-ischemic levels were only measured systemically. *P < 0.05 vs. systemic plasma levels, *P < 0.05 vs. plasma levels of the control group.

**Discussion**

Intestinal ischemia and reperfusion injury occurs as a continuum ranging from mild reversible post-ischemic organ dysfunction to permanent tissue damage characterized by intestinal necrosis. To evaluate mild and more severe intestinal ischemia, we used a rat model to investigate the effects of 20 and 40 minutes of SMA occlusion upon intestinal function (absorptive capacity and permeability), markers of (intestinal) cellular injury (LDH and \(\alpha\)GST) and intestinal morphology. Indeed, as expected, 20 minutes of intestinal ischemia showed only mild structural and functional intestinal changes, whereas 40 minutes of ischemia resulted in more pronounced intestinal injury, functional defects and morphological changes.

The focus of our study was to evaluate local (i.e. portal) activation of coagulation and fibrinolysis during intestinal ischemia and reperfusion. Endothelial injury, after ischemia and reperfusion, changes the vascular endothelium from an anticoagulant surface into a procoagulant surface by changing the synthesis and surface expression of endothelial proteins \(^1\). Perturbation of endothelial cells results in the induction of tissue-factor expression and suppression of thrombomodulin activity, leading to insufficiently
controlled thrombin generation and fibrin deposition. Indeed, in our experiments, the coagulation system in the intestinal microcirculation was activated after 20 or 40 minutes occlusion of the SMA. Marked thrombin generation, as evidenced by elevated thrombin-antithrombin complex levels and consumption of ATIII 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. This inhibition of fibrinolysis was preceded by a short-lasting increase in PAA, most probably released by endothelial cells upon injury. The resulting effect of fibrin generation and inadequate removal indeed resulted in intravascular fibrin deposition in the intestinal microcirculation. The fact that fibrin deposits were only demonstrated in a number of rat intestinal stainings, can be explained by the heterogeneity of intestinal injury after ischemia and reperfusion. This patchy distribution is supported by the variation in grade of intestinal injury (range 1-4) after 40 minutes ischemia and 3 hours of reperfusion, found with the histological grading score of Park-Chiu. Other authors have shown in mice that accumulation of fibrinogen onto the endothelial cell surface in the post-ischemic microvasculature of the intestine promoted platelet adhesion, early after the onset of reperfusion which may affect microvascular perfusion in postischemic intestine. Changes in the coagulation balance in portal blood were demonstrated even after 20 minutes ischemia, during which situation intestinal structure and function were largely intact, while 40 minutes of vascular occlusion demonstrated disrupted intestinal function and structure. Whether activation of coagulation and subsequent fibrin deposition after ischemia have any effect on intestinal function and structure is still uncertain, however, these data suggest that endothelial dysfunction precedes epithelial dysfunction.

Interestingly, the local intestinal 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 physiological anticoagulant pathways and inadequately balanced by a suppressed fibrinolytic system. This leads to widespread systemic intravascular fibrin deposition, eventually resulting in disseminated intravascular coagulation. In our study, the local intestinal changes in coagulation and fibrinolysis could be set off by endothelial injury and tissue-factor expression alone, or even by local inflammation caused by invasion of endotoxins (Figure 2).

Intestinal ischemia and reperfusion injury in itself may cause an increased permeability of the intestinal epithelial and endothelial barrier, which can lead to endotoxemia, thereby promoting the procoagulant state and intravascular fibrin deposition. We did not observe an increase in intestinal permeability after ischemia and reperfusion. In a comparable study of 20 and 40 minutes of intestinal ischemia, Sun and coworkers measured a two-fold increase of blood-to-lumen as well as lumen-to-blood permeability for albumin after 20 and 40 minutes of ischemia and 3 hours of reperfusion. The interpretation of these permeability studies is rather complex, because both the epithelial layer as well as the endothelial layer are restrictive to macromolecules such as albumin. We measured the clearance of Na$_2$Fl from the intestinal lumen as a direct marker of epithelial barrier function, and we did not observe any significant change of Na$_2$Fl clearance after ischemia. Therefore, in our study it seems unlikely that an intestinal barrier defect induced the procoagulant state. It is more likely, that intravascular coagulation activation is a consequence of endothelial injury.
Previous studies have shown that the activation of coagulation and fibrinolysis in the framework of a systemic inflammatory response are due to activation of pro-inflammatory cytokines. We previously showed that systemic activation of coagulation is mainly driven by interleukin-6 (IL-6), whereas for changes in anticoagulant and fibrinolytic pathways tumour necrosis factor-α (TNFα) can be held responsible \(^{27,28}\). Indeed, activation of the cytokine network (with a prominent role for IL-6 and TNFα) has also been demonstrated by other authors in models of intestinal ischemia and reperfusion \(^{29,31}\). This may demonstrate conjoined pathways in the cascade of inflammation and coagulation activation following intestinal ischemia and reperfusion.

In our model, the changes in coagulation and fibrinolysis in the systemic circulation were much less affected by intestinal ischemia and reperfusion than those in the local splanchnic (portal) circulation. Nevertheless, despite the rapid removal of different coagulation parameters (TAT, PAA and PAI) from the portal blood by the liver \(^{32,33}\), systemic changes of coagulation parameters were detectable. The coagulation parameters (TAT, FDP, PAA and PAI) in the systemic circulation showed significant changes corresponding to the changes found in the portal circulation upon ischemia and reperfusion, however, the changes in these parameters were less pronounced. These observations indicate that a systemic procoagulant state may indeed occur upon intestinal ischemia and reperfusion. This procoagulant state and microvascular obstructions may lead to delayed recovery of damaged tissue, or even may damage tissue at a site remote from the initial ischemic event.

In conclusion, we demonstrated that intestinal ischemia and reperfusion result in local generation of thrombin and subsequent conversion of fibrinogen to fibrin. Simultaneously, intestinal fibrinolysis is impaired, ultimately leading to intravascular fibrin deposition. These findings suggest that microvascular thrombotic obstruction plays a pivotal role in the pathogenesis of structural and functional intestinal injury induced by ischemia and reperfusion.

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