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Effects of anticoagulant treatment on intestinal ischaemia and reperfusion injury in rats

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Background: In recent years it has become increasingly clear that a cross-talk between the inflammatory response and blood coagulation exists, although many of the underlying mechanisms remain unclear. In the present study we investigated the potential anti-inflammatory properties of two different anticoagulant compounds, i.e. active-site inactivated FVIIa (FVIIai) and fondaparinux sodium, a selective FXa inhibitor, administered as pretreatment in a model of intestinal I/R in rats.

Methods: Endothelial barrier permeability was assessed using the vascular leakage of radiolabelled human serum albumin, tissue neutrophil sequestration was quantitated by myeloperoxidase (MPO) activity, and plasma levels of macrophage inflammatory protein (MIP)-2 were examined using an enzyme-linked-immuno-sorbent assay after 40 min of intestinal ischaemia and 6 h of reperfusion in the rat (n = 34). Pretreatment with FVIIai or fondaparinux sodium was administered 90 min before initiation of ischaemia.

Results: Endothelial-barrier permeability in all examined organs, myeloperoxidase activity in the lungs, and ileum and MIP-2 levels in plasma increased after intestinal I/R. Pretreatment with FVIIai decreased the endothelial barrier permeability and MPO activity in the ileum, and a tendency towards decreased permeability was also observed in the lungs. Fondaparinux did not affect the endothelial barrier permeability or MPO activity. Both FVIIai and fondaparinux decreased the MIP-2 levels in plasma after intestinal I/R.

Conclusions: Inhibition of the TF-FVIIa complex by FVIIai can attenuate inflammatory responses in connection with intestinal I/R-injury and could represent a potentially important therapeutic strategy for the prevention of organ dysfunction. Potential anti-inflammatory properties of fondaparinux and other inhibitors of FXa are not excluded and need further investigation.

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Key words: Active-site-inactivated FVIIa (FVIIai, ASIS); cytokine; endothelium; fondaparinux; inflammation; intestine; leukocyte recruitment; tissue factor.

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permeability, inflammatory cell migration and cytokine response (4, 5). In contrast to some of these findings it has been demonstrated that treatment with active-site-inactivated Fxa (FXai) only inhibited the coagulopathic response and failed to block the inflammatory and lethal response to LD\textsubscript{100} E. coli infusion in baboons (6). On the other hand, treatment with active-site-inactivated FVIIa (FVIIai) attenuated both the inflammatory as well as coagulopathic response, and it was hypothesized that the anti-inflammatory effect of FVIIai is elicited by inhibiting cytosolic Ca\textsuperscript{2+} flux by blocking the TF-VIIa interaction, which also may explain why FXai lacks anti-inflammatory properties, as it does not interfere with TF-FVIIa complex formation (7, 8).

Fondaparinux sodium is a synthetic pentasaccharide with antithrombotic activity through antithrombin III (AT III)-mediated selective inhibition of Fxa. By selectively binding to AT III, fondaparinux sodium potentiates (about 300 times) the innate neutralization of Fxa by AT III. This inhibition of Fxa via antithrombin results in effective inhibition of thrombin generation (9), potentially leading to attenuation of the inflammatory response.

The objectives in the present study were to investigate the potential anti-inflammatory properties of active-site-inactivated FVIIa (FVIIai) and the selective Fxa-inhibitor fondaparinux sodium in a murine intestinal ischaemia and reperfusion model, by evaluating endothelial permeability and tissue neutrophil recruitment, as well as plasma levels of macrophage inflammatory protein (MIP)-2, a potent neutrophil attractant belonging to the CXC-subfamily of chemokines, considered to be a functional murine homologue of human IL-8 (10, 11).

**Methods**

**Animals**

Adult male Sprague-Dawley rats weighing 250–300 g were fed standard rat chow (R\texttextsubscript{3}, Astra-Ewos, Sweden) and water ad libitum. The rats were allowed to acclimatize to our laboratory conditions for 6 days and were subjected to a regime of 12-h day/night cycle living in mesh stainless-steel cages (three rats/cage) at a constant temperature (22°C). The protocol was approved by the Institutional Review Board for Animal Research at Lund University. All animals were handled in accordance with the guidelines set forth by the Swedish Physiological Society.

**Induction of small intestinal ischaemia and reperfusion**

The rats were operated under aseptic conditions using anaesthesia with a mixture of ketamine hydrochloride (Ketalar\textsuperscript{R}, Pfizer AB, Täby, Sweden, 80 mg kg\textsuperscript{-1}) and xylazin hydrochloride (Rompun\textsuperscript{R}, Bayer Corp, Monheim, Germany, 10 mg kg\textsuperscript{-1}) administered s.c. Intestinal ischaemia and reperfusion (I/R) was induced by separating and clamping the superior mesenteric artery with an atraumatic clamp for 40 min, followed by 6 h of reperfusion. The restoration of blood flow to the intestines after declamping of the artery was ensured by visual control of the intestinal colour change. Sham operation was performed by separating the superior mesenteric artery without clamping for 40 min, followed by 6 h of sham reperfusion. Animals in the control group were not subjected to laparotomy or any other challenge. The animals were divided into five groups of six to eight animals in each; group A: control (no laparotomy), group B: sham operation, group C: I/R without treatment, group D: I/R and treatment with active-site-inactivated FVIIa (Novo Nordisk, Copenhagen, Denmark) 10 mg kg\textsuperscript{-1} i.p., group E: I/R and treatment with fondaparinux sodium (Sanofi-Synthelabo, Paris, France) 200 μg kg\textsuperscript{-1} s.c. Treatment to groups D and E was administered 90 min before the start of ischaemia. The dose and route of administration of FVIIai were selected on the basis of dose–response data provided by Novo Nordisk from previous studies on rats. The dose of fondaparinux sodium was selected partly based on human safety studies and partly on previous studies on rats from our group. During the period of ischaemia (40 min), the animals were anaesthetised and were then allowed to wake up during reperfusion. Ninety minutes before the end of the reperfusion period the animals were anaesthetised again and a catheter (Medical-Grade Tubing; 0.51 mm, OD 0.94 mm, Dow Corning Co., Midland, MI) was put into the femoral vein. Sixty minutes before the end of reperfusion, blood samples for measurements of haemostasis (prothrombin complex, activated partial thromboplastin time, fibrinogen, platelets) were taken and 1 ml of \textsuperscript{125}I-labelled human serum albumin (HSA, Isopharma AS., Kjeller, Norway) was injected. After 1 h of equilibration, 1 ml of blood was drawn from the femoral vein, followed by injection of \textsuperscript{51}Cr-labelled red blood cells (RBCs). The abdomen was then reopened and blood was withdrawn from the aorta and centrifuged for plasma analyses. A tracheostomy was performed and the animals were ventilated with room air using a ventilator (Rodent Ventilator UB 7025, Hugo Sachs Electronic, Harvard Apparatus, March –
by assuming that all $^{51}$Cr-labelled RBCs remained intravascularly, using the formula:

$$\text{albumin leakage index (ALI)} = \frac{^{125}\text{I counts per gram tissue}/^{125}\text{I counts per gram blood}}{\text{TBC}}$$

**Measurement of leukocyte recruitment**

After that the animals were exsanguinated and perfused, the left lung and the distal part of the ileum were harvested, frozen immediately in liquid nitrogen and stored at $-70^\circ$C until measurements. For MPO measurement we used a modification of the method described by Komatsu et al. (13). All chemicals were purchased from Sigma Chemical Co, St. Louis, MO. The samples were weighed (130–170 mg), put in 1 ml of ice-cold potassium phosphate buffer (20 mM, pH 7.4), homogenized (Homogenizer Omni 1000, Lambda Polynom, Sollentuna, Sweden) for 15 s on ice and then centrifuged at 20,000 r.p.m. for 15 min at 4°C (Sorvall RC-5B, Refrigerated superspeed centrifuge, Lambda Polynom). The supernatant was discharged by sonication in an ultrasonic bath (ULTRA sonik 28X, Neytech, Göteborgs Termometerfabrik AB, Gothenburg, Sweden) for 20 s and freezing ($-70^\circ$C) and thawing in order to permeate cellular membranes. Sonication, freezing and thawing were repeated once. The samples were then centrifuged at 20,000 r.p.m. for 15 min at 4°C. Of the final supernatant, 0.1 ml of blood was then kept at $-70^\circ$C until the assay was performed. Plasma levels of MIP-2 were determined using a sandwich enzyme-linked-immunosorbent assay (ELISA) specific for rat MIP-2 (DRG Instruments GmbH, Marburg, Germany). All samples were tested in duplicate. The plate was read in a microplate reader (Multiskan Plus, Thermo Labsystems OY, Helsinki, Finland) at
450 nm, and the MIP-2 concentration (pg ml⁻¹) in experimental samples was calculated from a standard curve.

**Statistics**
The data were analyzed using one-way analysis of variance by ranks (Kruskal–Wallis test), followed by the Wilcoxon rank sum test (Mann–Whitney U-test). Values are presented as means ±SD. Statistical significance was set at \( P < 0.05 \).

**Results**

**Endothelial permeability**
Albumin leakage index (ALI) increased significantly in the lungs and the ileum \((P < 0.05)\) following 40 min of intestinal ischaemia and 6 h of reperfusion as compared to both sham-operated animals and controls (Figs 1 and 2). In the colon, ALI also increased significantly after intestinal I/R as compared to controls \((P < 0.01)\), but did not reach statistical significance as compared to sham-operated animals \((P = 0.055; \text{Fig. } 1)\). Increased endothelial albumin leakage was also noted in the liver after intestinal I/R as compared to controls \((P < 0.01)\), but not as compared to sham-operated animals (Fig. 2).

Pretreatment with FVIIai decreased the albumin leakage index significantly in the ileum after intestinal I/R as compared to controls \((P < 0.01)\), but not as compared to sham-operated animals (Fig. 2). Pretreatment with FVIIai decreased the albumin leakage index significantly in the ileum after intestinal I/R as compared to I/R-challenged animals without treatment \((P < 0.05; \text{Fig. } 1)\). In the lungs, although not statistically significant \((P = 0.058)\), pretreatment with FVIIai resulted in a tendency towards decreased leakage (Fig. 2). In the colon and liver, pretreatment with FVIIai did not affect the albumin leakage index after 40 min of intestinal ischaemia and 6 h of reperfusion (Figs 1, 2). Pretreatment with fondaparinux did not affect the albumin leakage index significantly after intestinal I/R in any of the organs examined.

**Tissue myeloperoxidase activity**
Tissue leukocyte recruitment, measured as myeloperoxidase (MPO) activity (Fig. 3), significantly increased
in the lungs after intestinal I/R as compared to controls \( (P < 0.001) \) and sham-operated animals \( (P < 0.05) \). In the ileum, the MPO activity also increased after intestinal I/R as compared to controls \( (P < 0.05) \) and sham \( (P < 0.001) \).

Pretreatment with FVIIai significantly decreased the MPO activity in the ileum after intestinal I/R as compared to I/R-challenged animals without treatment \( (P < 0.001) \). In the lungs, pretreatment with FVIIai did not affect the MPO activity after intestinal I/R. Pretreatment with fondaparinux did not influence the MPO activity in any of the organs examined after 40 min of intestinal ischaemia followed by 6 h of reperfusion.

**MIP-2 plasma levels**

The plasma levels of MIP-2 (Fig. 4) increased significantly after intestinal I/R as compared to both controls \( (P < 0.001) \) and sham-operated animals \( (P < 0.01) \). Pretreatment with FVIIai, as well as with fondaparinux, reduced the I/R-induced increase in MIP-2 plasma levels significantly \( (P < 0.05) \) as compared to animals challenged with intestinal I/R without pretreatment.

**Haemostasis**

The prothrombin complex, expressed as international normalized ratio (INR), increased significantly \( (P < 0.001) \), but the activated partial thromboplastin time (APT-t) was unaffected after treatment with FVIIai, whereas fondaparinux did not influence any of these parameters, as compared to I/R-challenged animals (Table 1). The plasma levels of fibrinogen and blood levels of platelets did not differ significantly in any of the groups.

**Discussion**

The cross-talk between blood coagulation and the inflammatory response has gained increasing interest in recent years and it is now recognized that initiation of coagulation is an integral and consistent element of the response to inflammatory stimuli. In the present study, we focused on the potential anti-inflammatory properties of two different anticoagulant compounds, i.e. active-site inactivated FVIIa (FVIIai) and fondaparinux sodium, a selective FXa inhibitor, administered as pretreatment in a model of intestinal ischaemia and reperfusion in rats.

Intestinal I/R is considered to be an important initiating event in several pathophysiological conditions, e.g. shock, trauma, sepsis and pancreatitis, frequently leading to concomitant organ dysfunction and ultimately multiple organ failure (MOF).
Intestinal I/R-injury induced by superior mesenteric artery (SMA) occlusion represent an isolated gut injury model that not only result in intestinal tissue injury but also in secondary distant organ dysfunction. We have in previous studies demonstrated that intestinal I/R result in tissue injury and multiple organ dysfunction with increased permeability over the vascular endothelium, tissue leucocyte recruitment, consumption of protease inhibitors and increased levels of proinflammatory cytokines such as IL-1β and IL-6 (14–16). In the present study, we demonstrated that 40 min of intestinal ischaemia followed by 6 h of reperfusion resulted in an increased endothelial albumin leakage in the lungs and ileum, whereas the endothelial permeability in the colon and liver was less affected. This is in line with our previous studies and implies that the lungs and small intestines are the organs most vulnerable to tissue injury after intestinal I/R. In parallel with the endothelial permeability dysfunction, an increased neutrophil recruitment was observed in the lungs and in the ileum. Moreover, the plasma levels of MIP-2, which is considered to be a functional murine homologue of human IL-8 (10), also increased after intestinal I/R. MIP-2 is a potent neutrophil attractant and activator belonging to the CXC-subfamily of chemokines and is produced by a variety of cell types, including intestinal epithelium, macrophages, astrocytes and fibroblasts (11). Increased MIP-2 expression has been found to be associated with neutrophil influx in various inflammatory conditions and experimental models, including I/R-induced injury in the liver (17–19), kidney (20) and heart (21). It has been suggested that MIP-2 activates neutrophils by inducing CD11b/CD18 on the cell surface (22) and that the expression of MIP-2 in turn is regulated by oxygen-free radicals (OFRs) during I/R (21).

Treatment with active-site-inactivated FVIIa (FVIIai) 90 min before initiation of the ischaemic insult resulted in a significant reduction of the albumin leakage over the endothelial barrier in the ileum. A similar response was observed in the lungs and in the colon, though not reaching statistical significance in this study, with a rather small number of animals in each group. The MPO activity in the perfused ileum, reflecting both extravasated and remaining intravascular neutrophils, decreased after treatment with FVIIai, suggesting that inhibition of the TF-FVIIa complex have pivotal effects on the adhesion and migration of neutrophils. In parallel, plasma levels of MIP-2 decreased, indicating that one possible mechanism for the decreased tissue neutrophil recruitment may be due to the reduced influence of this neutrophil chemoattractant. In opposition to some other reports (23, 24), we did not note a corresponding effect in the lungs, where neutrophil recruitment was unaffected by FVIIai. However, data exist indicating that neutrophil adhesion mechanisms may have organ-specific as well as stimuli-specific differences and thus could inhibition of TF result in various effects on neutrophils in different tissues and after different inflammatory stimuli. It has, for example, been suggested that the CD11/CD18 complex, mediating neutrophil adherence in the systemic circulation, may occur by either CD18-dependent or CD18-independent mechanisms specific to the inciting stimulus in the pulmonary circulation (25). Furthermore, neutrophil adhesion per se may not completely explain neutrophil retention in pulmonary capillaries and increased cytoplasmic stiffness, induced by chemoattractants, could represent an important factor for the adhesive interactions to take place (26, 27). Nevertheless, this is the first study to our knowledge that demonstrates a specific reduction of organ injury after inhibiting the initiation of coagulation by TF after intestinal I/R and thereby supporting the current hypothesis that TF blockade could represent a potentially important therapeutic strategy for the prevention of organ dysfunction in hyperinflammatory states. FVIIai competitively inhibits binding of FVIIa to TF and suppresses both TF-VIIa signalling and coagulant activities (24). In the present study an evident anticoagulant effect was observed after treatment with FVIIai by a significant increase in the prothrombin complex ratio, whereas the APT-t, which does not depend on FVII activity, was unaffected.

### Table 1

Parameters of haemostasis.

<table>
<thead>
<tr>
<th></th>
<th>I/R</th>
<th>Control</th>
<th>Sham</th>
<th>FVIIai</th>
<th>Fondaparinux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin complex (INR)</td>
<td>1.5 ± 0.08</td>
<td>1.4 ± 0.06</td>
<td>1.5 ± 0.20</td>
<td>5.4 ± 1.76***</td>
<td>1.6 ± 0.16</td>
</tr>
<tr>
<td>APT-t (s)</td>
<td>42 ± 20.6</td>
<td>21 ± 2.5**</td>
<td>22 ± 3.5**</td>
<td>32 ± 11.4</td>
<td>38 ± 14.2</td>
</tr>
<tr>
<td>Fibrinogen (g l⁻¹)</td>
<td>3.0 ± 0.35</td>
<td>3.2 ± 0.21</td>
<td>2.9 ± 0.17</td>
<td>2.9 ± 0.50</td>
<td>2.9 ± 0.58</td>
</tr>
<tr>
<td>Platelets (× 10¹¹ l⁻¹)</td>
<td>716 ± 145</td>
<td>698 ± 60</td>
<td>815 ± 173</td>
<td>659 ± 212</td>
<td>807 ± 292</td>
</tr>
</tbody>
</table>

**P < 0.01, ***P < 0.001 vs. I/R.
Previous studies by other groups have demonstrated protective effects of TF blockade in sepsis-induced end-organ dysfunction in conjunction with an attenuated inflammatory response (7, 23, 24). An important issue to be answered is whether a later inserted therapy with TF blockade, i.e., when organ dysfunction has been established and therapy thereby more clinically relevant, result in the same protective effects. The mechanisms by which inhibition of the TF-FVIIa interactions lead to anti-inflammatory effects are not fully clear, but may include inhibition of cytosolic Ca$^{2+}$ flux (7, 8) and possibly inhibition of other intracellular signalling pathways, including phosphorylation of mitogen-activated protein kinases (MAPK) (28).

Fondaparinux did not influence endothelial permeability or neutrophil recruitment in this model of intestinal I/R, although plasma MIP-2 levels decreased. The explanation for this is unclear, but suggests that inhibition of the coagulation downstream of the TF-FVIIa complex formation just attenuates the inflammatory response to a certain degree, which may not be enough to protect from neutrophil recruitment and tissue injury in this I/R model. Moreover, neutrophil adhesion and migration mechanisms are complex and may not fully depend on MIP-2 effects, e.g., as in the lungs, as previously discussed. Other factors that have been implicated as mediators in I/R-induced neutrophil recruitment and tissue injury, such as OFRs, platelet activating factor (PAF) and leukotriens (29), may not be influenced unless TF-FVII complex formation is inhibited. The synthetic pentasaccharide fondaparinux is the first of a new class of antithrombotic agents that selectively inhibit FXa via binding to antithrombin III (AT III), which in turn inhibits thrombin generation, without any direct effect on thrombin activity (30–33). Several clinical studies have demonstrated a reduced risk of venous thromboembolism with fondaparinux after major orthopaedic surgery, without an increased bleeding rate. It has been suggested that FXa may function as a mediator of acute inflammation (34), and it has furthermore been implied that the active site of FXa is involved in FXa-stimulated cytokine production (4). In line with the present study, FXa was found to stimulate C-X-C chemokine CINC production by macrophages after hepatic I/R-injury and that selective inhibition of FXa inhibits this production (35). Mechanism could involve an inhibition of FXa-induced TF expression or blocking of thrombin generation (36). Although enzymatic activity of FXa seems to be needed for cytokine production (4), it has been demonstrated that administration of FXa blocked in the active centre (FXai) only inhibit the coagulopathic pathway induced by infusion of E. coli, but fail to block the lethal effects of the bacterial infusion (6). Also active site-independent FXa-mediated inflammation have been demonstrated (34) and the exact biological mechanisms of factor Xa-induced proinflammatory responses remain unclear and need to be further investigated. The lack of a protective effect by fondaparinux in the present study on tissue injury and tissue neutrophil recruitment, although plasma levels of MIP-2 decreased, might be due to an insufficient dose administered to the animals. Although it is reported that fondaparinux does not affect coagulation parameters such as APT-t and prothrombin complex (INR) in humans using a dose of 2.5 mg once-daily for prophylaxis of venous thromboembolism (33), sufficient inhibition of FXa should result in increases in both these parameters. Limited dose–response data exist on fondaparinux used in rats when focusing on potential anti-inflammatory effects and it is not known whether an anticoagulatory effect visible on APT-t and prothrombin complex (INR) is necessary to reach an effect on the inflammatory response. We do not exclude that an anti-inflammatory effect of fondaparinux could exist and further dose–response studies are needed to elucidate this.

In conclusion, the present study provides novel information by demonstrating that inhibition of the TF-FVIIa complex formation by FVIIai can attenuate inflammatory responses, such as endothelial barrier dysfunction, tissue neutrophil recruitment and chemokine production in connection with intestinal I/R-injury. Selective inhibition of FXa did not result in a corresponding effect, although decreased plasma levels of MIP-2 were observed. Anticoagulant treatment in hyperinflammatory states could represent a potentially important therapeutic strategy for the prevention of organ dysfunction, and further studies are needed to clarify the effects and mechanisms of the cross-talk between coagulation and inflammation.

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References


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