ANNALS OF GASTROENTEROLOGY 2010, 23(3):180-186

*Original article*

# Treatment with active site inhibited factor VIIa - effects on inflammation in experimental acute pancreatitis

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## SUMMARY

**Objective: In critical illness, e.g. severe acute pancreatitis (SAP), an extensive cross-talk between inflammation and coagulation seems to exist. In previous studies in rats with SAP, we have demonstrated an anti-inflammatory effect following pre-treatment with active-site inhibited factor VIIa (fVIIai). In the current experiment the effect of treatment with fVIIai is studied, i.e fVIIai administered first after the induction of SAP in rats. Material and methods: AP was induced by infusion of taurodeoxy-cholic acid (5%) into the common bile duct. FVIIai was administered 30 min and 90 min, respectively, after the onset of AP. One group received no treatment. Non-operated and sham-operated rats were used as controls. The effect was evaluated after 6 hours by myeloperoxidase (MPO)-content in the lungs, ileum and pancreas, and by plasma levels of interleukin (IL)-6 and macrophage inflammatory protein (MIP)-2. The effect on coagulation was measured by PT, APTT, D-dimer and fibrinogen. Results: In rats receiving fVIIai 30 min after induction of AP, MPO-levels were significantly lower in the lungs, and plasma concentrations of MIP-2 showed a trend towards reduction as compared to non-treated rats with AP. There was also a tendency towards lower IL-6 levels in plasma. No effect was seen on MPO-levels when fVIIai was given after 90 min, but there was a tendency towards lower MIP-2 and IL-6 levels. MPO in the ileum was low in all groups. MPO levels in the pancreas were not affected by treatment. Conclusions: Active site-inactivated factor VIIa, administered as early treatment after induction of taurodeoxycholate-induced AP in rats, seems to possess anti-inflammatory effects, reflected by a reduction of the influx of neutrophils in the lungs, and a tendency towards reduced plasma levels of MIP-2 and IL-6. These effects were less evident when fVIIai was administered later on during the course of disease.**


**Key words:** active-site inactivated factor VIIa, acute pancreatitis, coagulation, inflammation, myeloperoxidase, neutrophils, rat

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## INTRODUCTION

Acute pancreatitis (AP) is most often a mild, self-limiting disease. In some cases it may, however, progress to a pronounced systemic inflammatory response with the potential development of multiple organ dysfunction, associated with a substantial risk of mortality. In critical illness, e.g. severe acute pancreatitis (SAP), an extensive cross-talk between inflammation and coagulation seems to exist. SAP is accompanied by alterations in coagulation, in the most extreme cases resulting in disseminated intravascular coagulation, with simultaneous bleeding and formation of thrombi.

With this background it seems plausible to expect effects on inflammation by the administration of anticoagulants, and this has previously been shown in various experimental and clinical settings.[1-4] One of the key explanations to this cross-talk is probably the family of proteases, with capacity to both initiate inflammatory events and activate coagulation.[5-7]

Focusing on AP, disturbances of microcirculation with reduced blood flow in the pancreas may be one of the initial mechanisms in the development of AP.[8] The formation of microthrombi resulting in ischemia has been suggested to play a crucial role in the development of AP, emphasizing the possibility of anticoagulation to act as a potential therapeutic tool in AP. Clinical evidence, such as micro-

thrombi and atheromatous emboli in the pancreatic artery of patients with AP may support pancreatic infarction, or at least imply the importance of pancreatic hypoperfusion as an etiological factor in the development of pancreatitis.[9,10] Apart from ameliorating inflammation by improving microcirculation, anticoagulants may have direct anti-inflammatory effects in AP, irrespective of their effects on coagulation, by their ability to induce intracellular signalling through protease-activated receptors (PARs).[5-7]

Focusing specifically on previously studied effects of anticoagulants in AP, heparin and low-molecular weight heparin have been thoroughly investigated. Both substances have in many experimental and minor clinical studies been able to decrease the incidence of post-ERCP pancreatitis. However, in randomized placebo-controlled trials on the potential prevention of post-ERCP pancreatitis, the results have been discouraging.[11,12] Aspirin, as well as heparin, may prevent oedema and ameliorate the decrease in lung compliance in rat models of severe AP.[13,14] The ability of activated protein C (APC) to reduce mortality in severe sepsis has evoked hope to get similar effects in AP. So far, the results from experimental SAP in rats are contradictory. Three studies revealed a reduced inflammatory response by treatment with APC, while no beneficial effects on inflammatory parameters were noted in a recent study.[15-18]

We have, in previous studies on AP in rats revealed anti-inflammatory effects by pretreatment with active site inhibited factor VII (fVIIai). The inhibition of the inflammatory response was demonstrated by a decreased infiltration of leukocytes in remote organs (lungs and ileum), and reduced plasma levels of proinflammatory cytokines (IL-6 and MIP-2).[1] We have also detected a decreased activation of the transcription factor nuclear factor (NF-κB) in remote organs following fVIIai pre-treatment,[2] which indicate that NF-κB represents one of the pathways where fVIIai exerts its anti-inflammatory effects.

Experimental data evaluating the effectiveness and the influence on the inflammatory response by fVIIai administered as treatment in AP, i.e. administration after the induction of AP, has not been published previously.

The aim of the present study was to investigate if treatment with fVIIai, given after induction of AP, has any effect on the local and systemic inflammatory response in a rat model of SAP. The inflammatory response was measured by myeloperoxidase (MPO) activity in the pancreas, lung and ileum, as a parameter of tissue neutrophil recruitment, and by plasma concentrations of the proinflammatory interleukin 6 (IL-6) and the chemokine macrophage inflammatory protein (MIP)-2. MIP-2 is known to be a potent neutrophil chemoattractant. The effects on coagulation were evaluated by plasma levels of PT, APTT, D-dimer and fibrinogen. As control groups non-operated rats and sham-operated rats were used.

## MATERIALS AND METHODS

### *Animals*

Adult male Sprague-Dawley rats weighing 200-250g were fed standard chow (R3, Astra-Ewos, Sweden) and water ad libitum. The rats were allowed to acclimatise to our laboratory conditions for six days, subjected to a regime of 12 hours day/night cycle in mesh stainless-steel cages (three rats/cage) at constant temperature (22°C). The protocol was approved by the Swedish Animal Welfare Agency. All animals were handled in accordance with the guidelines set forth by the Swedish Physiological Society.

### *Induction of acute pancreatitis*

The operations were performed using anaesthesia with a mixture of ketamine hydrochloride (Ketalar, Parke-Davis, Warner Lambert Nordic AB, Solna, Sweden; 50 mg/ml) and xylazin hydrochloride (Rompun vet., Bayer AB, Gothenburg, Sweden; 20 mg/ml). Acute pancreatitis was induced by intraductal infusion of sodium taurodeoxycholate (5%) in the common bile duct, as described in previous studies.1 After surgery, buprenorphine hydrochloride (22.5 μg/kg; Temgesic®, Schering-Plough AB, Stockholm, Sweden) was administered. In the sham-group laparotomy was performed and the common bile duct was dissected, but neither cannulation, nor infusions were conducted.

### *Experimental design*

The animals were divided into five different groups with 6-8 rats in each group. In all animals, except for the control group (non-operated, non-treated) and the sham-operated group, acute pancreatitis was induced.[1] The treatment groups were given intraperitoneal injections of fVIIai (10 mg/kg; Novo Nordisk, Copenhagen, Denmark) 30 minutes or 90 minutes after the induction of AP. At the end of the experiment, blood was drawn from the abdominal aorta and collected in EDTA tubes (for IL-6 and MIP-2 analyses) and citrate tubes (for analyses of coagulation parameters). The organs were perfused with phosphate buffered saline through a catheter in the portal vein. Lungs, ileum and pancreas were harvested 6 hours after the induction of acute pancreatitis in all animals, a time-point at which AP is in a fulminant state of the disease in this model.[1] Tissues were snap fro-

zen in liquid nitrogen and stored in -80 °C until analyses were performed. Blood samples were kept on ice until centrifugation.

### *Measurement of myeloperoxidase (MPO) activity*

MPO activity in tissue samples from pancreas, lung and ileum, was analysed as described in detail previously,[1] and the method is therefore only briefly outlined here. Tissues were homogenised in phosphate buffered saline and centrifuged. The pellet was repeatedly washed, sonicated and frozen. Phosphate buffered saline, $H_2O_2$, hexadecyl trimethylammonium bromide, and the substrate, 3,3',5,5'-tetramethylbenzidine were added to the supernatant. After incubation, the reaction was stopped and the change in absorbance measured. The MPO activity is considered proportional to the amount of neutrophils in the tissue.

### *IL-6*

Plasma was obtained by centrifugation (3400*g*, at 4°C, for 10 minutes). Plasma concentrations of IL-6 were quantified by sandwich enzyme-linked-immunosorbent assays (ELISA, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions, and measured spectrophotometrically at 450 nm.

### *MIP-2*

Plasma was obtained by centrifugation (3400*g*, at 4°C, for 10 minutes). Plasma concentrations of MIP-2 were quantified by sandwich enzyme-linked-immunosorbent assays (ELISA, R&D Systems, Minneapolis, USA) according to the manufacturer's instructions, and measured spectrophotometrically at 450 nm.

### *Coagulation parameters*

PT, APTT, D-dimer and fibrinogen were analysed by the department of Clinical Chemistry and Pharmacology, Lund University Hospital, by automated analyses in Sysmex CA-7000 (Sysmex Deutschland GmbH).

### *Statistics*

For all comparisons the non-parametric Mann-Whitney test were used. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, Ill, USA). No corrections for multiple comparisons were made. Outliers were defined as values more than 1.5 times the interquartile range. Outliers are included in all calculations, but excluded from the figures.

## RESULTS

### *MPO*

#### *Lungs*

In the group receiving fVIIai-treatment 30 minutes after induction of AP, the MPO activity in the lungs was significantly lower in the treated groups compared to the non-treated AP group. (Fig. 1). No effect of treatment with fVIIai after 90 min was detected (data not shown).

#### *Ileum*

In the ileum no statistically significant increase of MPO activity was detected in the non-treated AP group, compared to the sham group or to the control group. The MPO levels were very low in all groups. No effect of treatment could be seen, neither when given after 30 min, nor after 90 min (data not shown).

#### *Pancreas*

Treatment with fVIIai showed no effect on the MPO activity in the pancreas (data not shown).

### *IL-6*

The IL-6 concentration in plasma was higher in the non-treated AP group, as compared to the sham group. A tendency towards decreased IL-6 levels was seen in the group treated with fVIIai 30 minutes after induction of AP ($p=0.056$) as compared to the non-treated AP group (Fig. 2), and similar results were noted when treatment with fVIIai was given after 90 minutes ($p=0.055$).

### *MIP-2*

The MIP-2 concentration was higher in the non-treated AP group as compared to the sham group. There was a trend towards lower MIP-2 levels in the group receiving fVIIai after 30 min ($p=0.063$) as compared to the non-treated AP group (Fig 3). A similar result was seen when FVIIai was given after 90 minutes ($p=0.067$) (data not shown).

### *Coagulation parameters*

None of the analysed coagulation parameters (PT, APTT, D-dimer and fibrinogen) were affected in the non-treated rats with AP as compared to the control group or the sham group. PT levels increased when fVIIai was administered (Fig. 4), whereas APTT, D-dimer and fibrinogen were unaffected by fVIIai (data not shown).

## DISCUSSION

In this study we confirm the results from our previous studies in which fVIIai was administered as pre-treatment prior to the induction of AP, indicating that fVIIai has sys-

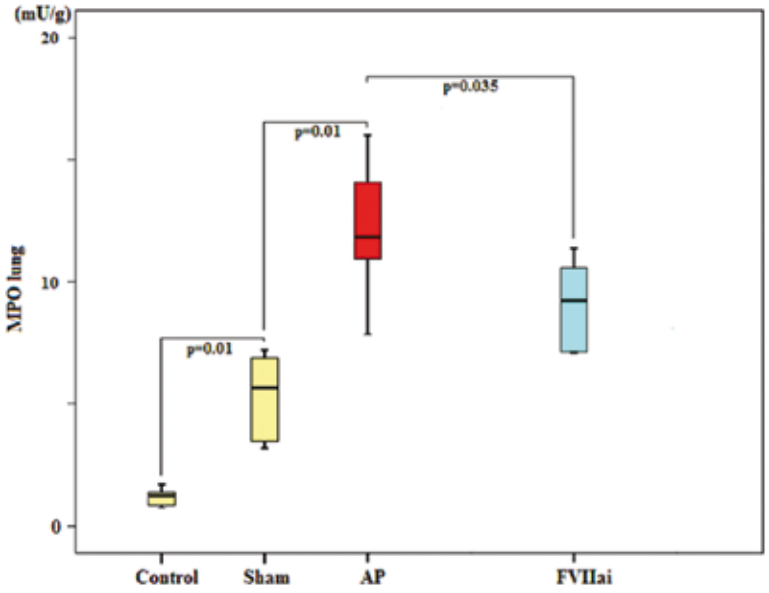


**Figure 1.** MPO activity in the lungs (mU/g tissue). FVIIai treatment after 30 min.

temic anti-inflammatory properties in this model of SAP in the rat.[1,2] In this study, early treatment with fVIIai ameliorated inflammation, though less efficiently compared to when given as pre-treatment. Evidently fVIIai is being less effective when administered at a more pronounced stage of the disease. These are novel findings implying that the potential therapeutic window for the use of fVIIai is limited in time, but provides effects if administered early during the course of disease.

When fVIIai was given during the early phase of the disease, i.e. 30 minutes after the induction of AP, this resulted in a decreased MPO activity, reflecting a reduced recruitment of leukocytes in the lungs. In our previous pre-treatment study,[1] we noted a small raise in MPO activity in the ileum 6 hours after induction of AP, which could not be confirmed in the present study. The multiple organ dysfunction associated with AP tend to follow a certain order, where the lungs will be the first remote organ to be af-

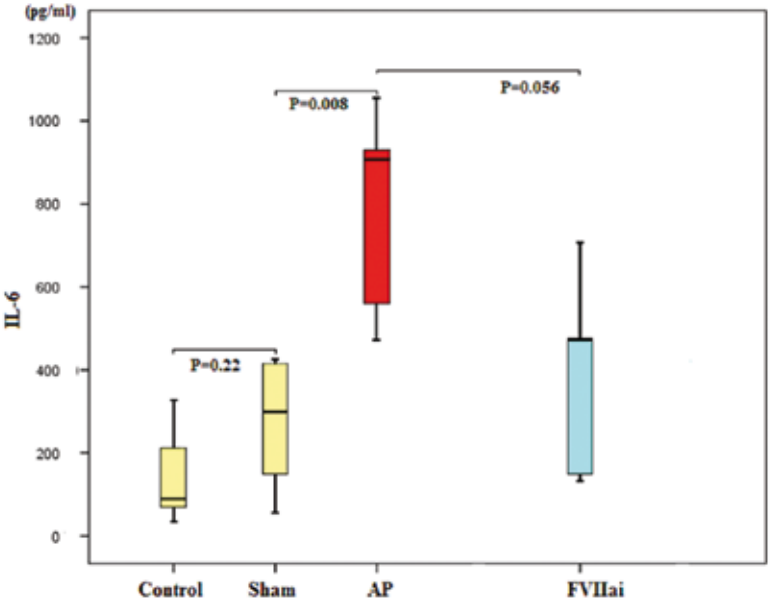


**Figure 2.** Concentrations of IL-6 (pg/ml) in plasma. FVIIai treatment after 30 min.

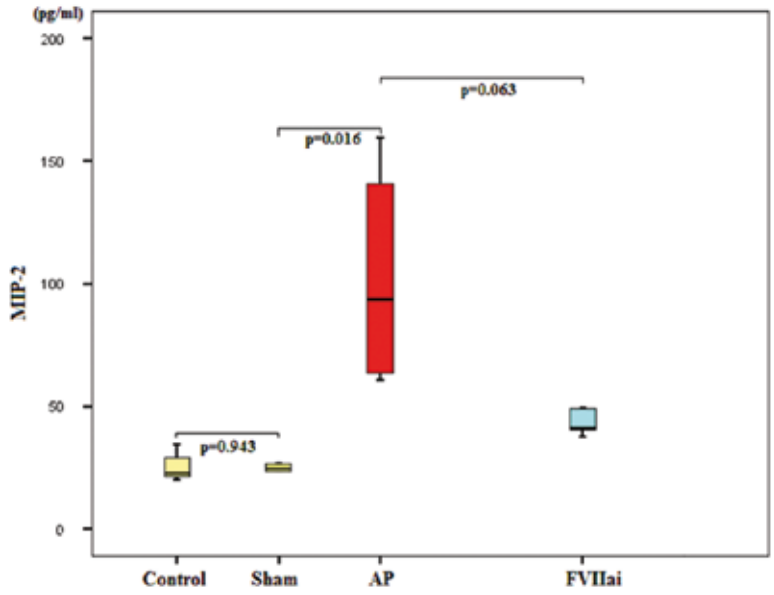


**Figure 1.** MPO activity in the lungs (mU/g tissue). FVIIai treatment after 30 min.

fected, while e.g. ileum and kidneys will be affected later during the course of the disease. A later time point would probably result in a more true reflection of the intestinal inflammation. At the chosen time point, there was, however, a trend towards a decrease in the plasma concentration of the pro-inflammatory chemokine MIP-2, and there was a tendency towards lower levels of IL-6, confirming a reduction in the systemic inflammation. No effect was seen on pancreatic MPO activity. This is, of course, not what would be expected. The explanation may be the character of the model. In this model, AP is induced by intraductal infusion of sodium taurodeoxycholate, which may act as a tissue detergent and dissolve both the pancreatic duct and adjacent tissue. Because of the extensive tissue destruction and subsequent inflammation, it is unlikely to get an effect of treatment in the pancreas, although it

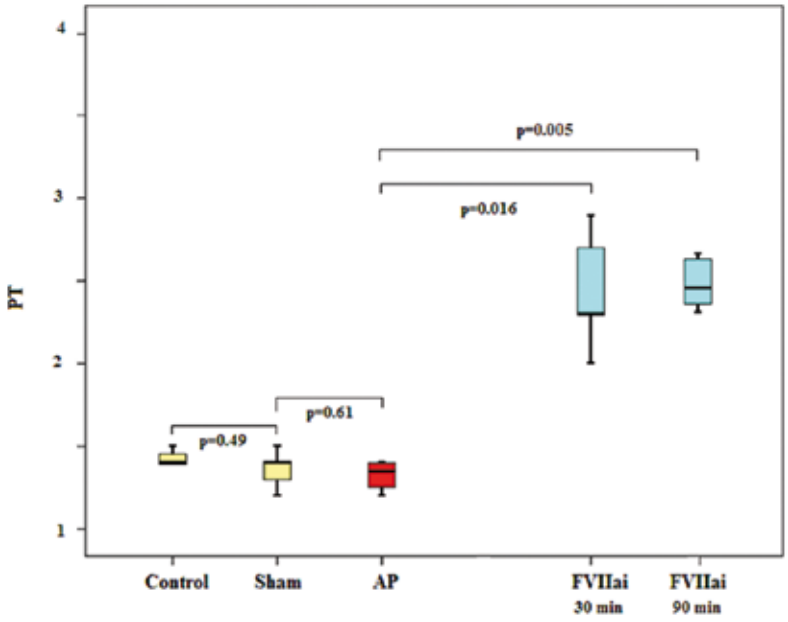


**Figure 2.** Concentrations of IL-6 (pg/ml) in plasma. FVIIai treatment after 30 min.

may still be effective in remote organs. A different model, with less extensive destruction of pancreatic tissue, such as e.g. cerulein-induced pancreatitis, may reveal beneficial effects of fVIIai treatment in the pancreas per se. Another explanation may be the role of PAR:s, a family of G protein-coupled transmembrane receptors. In acute pancreatitis PAR-2 is of particular interest as it is located on both acinar and ductal cells and is activated by trypsin. It has been shown in pancreatic tissue that PAR-2 is involved in cell secretion and arterial tonus regulation under physiological conditions. PAR-2-stimulation seems to have protective effects on inflammation in the pancreas itself, but may actually worsen the systemic inflammation.[19] To make it even more complex, PAR-2 seems to exert contrasting model-specific effects in experimental AP.[20] Of specific interest to our study is that factor VIIa is able to induce signalling through PAR-2.[21] Hypothetically fVIIai may act by blocking PAR-2 signalling, thereby reducing the systemic inflammatory response, but enhance inflammation within the pancreas. Interestingly, in a cell culture model with LPS-stimulated monocytes fVII is able to induce gene-expression of IL-6 and IL-8, while fVIIai has no effect on these genes.[22]

Concerning the association between anti-inflammatory and anti-coagulant effects, the aim is to choose a dose of fVIIai which exhibits a strong inhibition of inflammation without dangerously increasing the risk of bleeding. In this study we used a dose similar to doses used in other models testing fVIIai, such as an ischemia-reperfusion model in rats.[23] The effect on coagulation, reflected as a raise in PT, is dose-dependent, increasing with higher doses of fVIIai. No overt intraabdominal bleeding was noted in the treated animals, but they seemed to bleed more from the incision before closure of the abdominal wall and the abdominal fluid in the treated animals was light red. The other parameters used to evaluate the impact on coagulation (APTT, D-dimer, fibrinogen) are not affected, neither by AP, nor by treatment. This indicates that the rats in this model do not suffer from DIC or other severe coagulation abnormalities, at least not during the time period in this study, i.e. six hours after the induction of AP. The absence of coagulopathy further supports the hypothesis that it is unlikely that fVIIai exerts its anti-inflammatory effect only by inhibiting the formation of microthrombi.

The anti-inflammatory effect of treatment with fVIIai after 30 minutes cannot be reproduced when fVIIai is administered during the more fulminant state of the disease, i.e. in this case 90 minutes after the induction of pancreatitis. No statistically significant effect could be demonstrated on pancreatic or remote organ MPO activity. A tendency towards lower levels of IL-6 and MIP-2 was seen, though not really reaching statistical significance ($p=0.055$ and $p=0.067$, respectively). The lack of evident effect in the fulminant stage of the disease may reflect that the inflammatory response is regulated by different pathways in the early and during the later phase. One explanation may be that the anti-inflammatory effect of fVIIai is mediated predominantly by inhibiting activation of monocytes. This would explain why fVIIai will be efficient only when given as early treatment, when the monocytes are considered to be important actors in the development of the inflammatory response. Another explanation may be the expression of TF and fVIIai. It is known from the clinical situation that plasma concentrations of TF are increased in patients with SAP.[24] This has also been shown experimentally. In rabbits subjected to chenodeoxycholic acid-induced AP, both TF and fVII coagulant activity peak at two hours after infusion.[25] This could explain the loss of anti-inflammatory effects of fVIIai at later stages, by increased amounts of endogenous TF and fVII competing with the inhibitor. Later time points were not evaluated in this experiment, but it is likely that an even weaker effect would be seen, as many inflammatory processes and pathways gradually are initiated, many of which are not regulated by proteases.

Despite the fact that the experimental setting may seem far from the clinic, it is still interesting to note that fVIIai demonstrates some anti-inflammatory properties, even when administered after the induction of AP. Most substances with therapeutic effects in the setting of experimental AP have been efficient primarily when given as pretreatment, i.e. before the induction of AP. The results of the present study reflect a potential therapeutic window for fVIIai during the early course of experimental AP in this model, whereas fVIIai given in the fulminant state of the disease does not influence the systemic inflammatory response.

More studies on the mechanisms by which fVIIai reduce inflammation in SAP are warranted, including investigation of which cells and which signalling pathways are affected. The risk of bleeding complications must also be taken into consideration. One must argue that taurodeoxycholate-induced severe pancreatitis in the rat is quite far from the clinical setting. Nevertheless, these findings are important, as many substances are effective only when given as pre-treatment in experimental AP, and do not show any effect at all once the AP has been initiated.

So far, the conclusion from our studies is that fVIIai shows interesting anti-inflammatory properties in this model of SAP. However, it is too early to predict whether

the concept of active-site inactivated factor VIIa warrants a role in future management of patients with AP, used alone or in combination with other types of intervention.

## ACKNOWLEDGEMENTS

The authors wish to thank Novo Nordisk, Copenhagen, Denmark, for kindly providing fVIIai.

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