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Potential effects of PKC or protease inhibitors on acute pancreatitis-induced tissue injury in rats

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ABSTRACT

Background: Acute pancreatitis (AP) is still one of the severe diseases that cause the development of multiple organ dysfunction with a high mortality. Effective therapies for AP are still limited, mainly due to unclear mechanisms by which AP initiates both pancreatic and extrapancreatic organ injury. **Methods**: Protease inhibitors (aprotinin, pefabloc, trypsin inhibitor) and PKC inhibitors (polymyxin B, staurosporine) were administrated 30 minutes before induction of AP in rats. To investigate the pancreatic, systemic and lung inflammatory response and injury, plasma IL-6 and IL-10, pancreatic and pulmonary myeloperoxidase (MPO) levels, pancreatic protease activity and phospholipase A_2 (PLA_2) activity in ascites were measured 3 and 6 hours after AP induction. Results: Pretreatment with protease inhibitors significantly prevented from AP-increased plasma levels of IL-10, pancreatic and pulmonary levels of MPO, pancreatic protease activity and the catalytic activity of PLA_2 in ascites. PKC inhibitors significantly reduced pancreatic and pulmonary levels of MPO and pancreatic protease activity. Conclusion: Inhibition of proteases in AP may be helpful in ameliorating the inflammatory reaction in both pancreatic and extrapancreatic tissues, where neutrophil involvement may be regulated by PKC and proteases.

Key words: Acute pancreatitis, protein kinase C, protease, inhibitors.

Introduction

It is generally believed that acute pancreatitis (AP) results from the intrapancreatic activation of digestive enzyme zymogens (Saluja et al., 1999), even though there is still the lack of understanding pathophysiological mechanisms of AP. Pancreatic proteases have been suggested to be one of critical factors leading to the development of pancreatitis-associated with lung injury (Hartwig et al., 1999). Experimental studies demonstrated that protease inhibition could improve the severity of the disease (Hartwig et al., 1999; Saluja et al., 1999; Singh et al., 2001), but the efficacy of protease inhibition should be furthermore clarified in clinical trials (Pelagotti et al., 2003). The effects of protease inhibitors may be related with the etiology of pancreatitis. For example, an intracellular trypsin inhibitor, Gabexate mesilate, significantly decreased the incidence of pancreatitis induced by post-endoscopic retrograde cholangiopancreatography (Cavallini et al., 1996).

Protein kinase C (PKC), a family of serine/threonine kinases with about 11 different isotypes (Schechtman and Mochly-Rosen, 2001), is another factor probably involved in intracellular signaling in pancreatitis-induced primary and secondary organ injury. It was found that PKC activation and intracellular Ca^{2+} mobilization, two major signaling pathways, mediate cholecystokinin-induced activation of nuclear factor Kappa B (NF-κB) in isolated pancreatic acini or lobules (Han and Logsdon, 2000). Overactivation of NF-κB could increase pancreatic sensitivity to the inflammatory response through signaling pathways involving novel or atypical PKC isoforms (Gukovskaya et al., 2004). Activated PKC can not only initiate NF-kB activation, but also induce the production of inflammatory mediators (Lin et al., 2001). PKC could be proteolytically activated by a variety of proteases such as calcium-dependent

proteases and trypsin-like serine proteases (Chakraborti et al., 2004; Hashimoto and Yamamura, 1989).

The clinical therapeutic strategies in AP have so far been mainly directed at supportive critical care due to a lack of knowledge as regards underlying pathophysiological events. Therefore, the present study investigates the potential effects of inhibitors of proteases and PKC on local, systemic, and distant organ inflammatory responses in experimental AP.

Materials and methods

Animals: Adult male Sprague-Dawley rats, weighing about 250 g, were fed standard rat chow (R_3, Astra-Ewos, Södertälje, Sweden) and water ad libitum. The rats were allowed to acclimatize to our laboratory conditions for 5 days and were subjected to a regime of 12 hours day/night cycle living in mesh stainless- steel cages (3 rats/cage) at constant temperature (22^{o}C). The protocol was approved by the Animal Ethics Committee at Lund University. All animals were handled in accordance with the guidelines set forth by the Swedish Physiological Society.

AP induction: The rats were operated on under aseptic conditions using an intramuscular mixture of ketamine (70mg/kg) and rompun (25mg/kg) (Sigma Aldrich, Stockholm, Sweden) anesthesia. AP was induced by the intraductal administration of 0.2 ml of 0.025 M glycylglycin-NaOH buffer, PH 8.0, containing 5% sodium taurodeoxycholate sterilized at 100 ^{o}C for 20 min, infused by use of an infusion pump at a speed of 0.04 ml/min, following clamping of the proximal end of the common bile duct and cannulating the biliary- pancreatic duct by a thin polyethylene catheter (0.66mm OD, Portex Ltd., Hythe, Kent, UK). Sham operation (controls) included laparotomy and separation of the common bile duct similar to what was performed in the experimental group, but without bile injection.

Experimental design: The rats were randomly allocated into seven groups: 1) sham-operated animals pretreated with saline, 2) AP animals pretreated with saline, 3) AP animals pretreated with aprotinin (1mg/kg), 4) AP animals pretreated with pefabloc (10mg/kg), 5) AP animals pretreated with trypsin inhibitor (10mg/kg), 6) AP animals pretreated with polymyxin B (50 mM/kg), and 7) AP animals pretreated with

staurosporine (0.1mg/kg). These inhibitors were selected on basis of their various functions. Aprotinin is a reversible enzyme-inhibitor, while pefabloc is an irreversible enzyme-inhibitor, although they all belong to the group of serine protease inhibitors. In addition, Aprotinin possess an effect on inflammation and the trypsin inhibitor mainly inhibits trypsin. Polymyxin B and staurosporine have a different pharmacological site of PKC, i.e. PKC/phospholipid interaction and the ATP binding site of PKC, respectively. Staurosporine is more potent than Polymyxin B. Each group included 16 animals and animals were intraperitoneally injected 30 minutes prior to sham operation or induction of AP. The animals were terminated three or six hours after sham operation or induction of AP by an overdose of anesthesia (n=8 rats/time point/group). Ascites samples were collected with a disposable tube containing EDTA Na (7.7 mM, pH 7.4) and prostaglandin E1 (PGE1) (1.5 g/ ml). They were centrifuged at 2000 X g for 15 min to remove the cells, and the supernatants will be analyzed for PLA_2 activity. Blood samples were obtained by puncture of the aorta and plasma was collected after centrifugation (3000 rpm) for 10 minutes. Samples from the pancreas and lungs were rapidly collected after the perfusion with Phosphate Buffered Saline (PBS), immediately frozen in liquid nitrogen, and stored at -80ºC until processing.

Measurements of interleukins (IL): Plasma levels of IL-6 and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA). Antibodies specific for rat IL-6 and IL-10 (Pharmingen, San Diego, CA, USA) were coated onto the wells of the microtiter strips (NUNC, Copenhagen, Denmark) and the samples including standards of known rat IL-6 and IL-10 were pipetted into the wells, incubated and washed. Intensity of the color was determined at 405 nm.

Myeloperoxidase (MPO) activity: The tissue samples from the pancreas and lungs (100-200 mg) were weighed, put in ice-cold potassium phosphate buffer (20 mmol/l; pH 7.4) and homogenized for 30 seconds. The suspension was centrifuged at 20000 rpm for 15 minutes at 4°C, after which the supernatant was discarded. The precipitates were rehomogenized with PBS 50 mmol/l containing 0.5 % hexadecyltrimethylammoniumbromide and ethylenediamine tetra-acetic acid 10 mmol/l, followed by sonicating, freezing, thawing and homogenizing twice. The reaction was terminated with sodium acetate (0.2 mol/l; PH 3.0), after incubation for 3 minutes at 37°C with a reaction solution containing 0.5% hexadecyl-trimethyl-ammonium-bromide, 3,3,5,5-tetramethyl-benzidine (1.6 mmol/l) and hydrogen peroxide 0.3 mmol/l in PBS (80 mmol/l; PH 5.4). MPO activity was counted as the change in absorbance at 655 nm per minute.

Protease activity: The pancreatic protease activity was assessed by using the EnzChek® Phosphate Assay Kit (Molecular Probes Europe BV, Leiden, Netherlands). Digestion buffer was diluted 20 times as recommended. BODIPY casein working solution was firstly diluted with PBS and then prepared with the digestion buffer. 100ml of each homogenized pancreatic sample were added into a fluorescence microplate, followed by the addition of 100ml of the BODIPY casein working solution. The plate was incubated for one hour at room temperature and protected from light. The range of enzyme response was determined by reading the fluorescence in a fluorescence microplate reader. The fluorescein filters were set as excitation = 485 ± 12.5 nm and emission = 530 ± 15 nm.

PLA_2 catalytic activity: The catalytic activity of PLA_2 in ascites was measured according to the method of Yoshikawa et al. (Yoshikawa et al., 1999). Briefly, the substrate was used in the form of mixed micelles of 1 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 3 mM sodium deoxycholate. The assay mixture contained 5 mM $CaCl_2$, 1 mg/ ml fatty acid free BSA, 150 mM NaCl, and 100 mM Tris-HCl (pH 8.0). The reactions were initiated by addition of ascites samples to the assay mixture. The reaction was carried out at 40^oC for 30 min and then stopped, and the mixture was subjected to extraction according to Dole's extraction system followed by silicic acid treatment. PLA_2 activity was determined by measuring 9-anthryldiazomethane (ADAM)-labeled free fatty acid with HPLC.

Statistics: The data were analyzed using unpaired Student's t-test or a nonparametric test (Mann-Whitney U test), where appropriate, after ANOVA analysis. Values are described as means ± SEM. A probability of <0.05 was considered significant.

Results

Induction of AP caused significant increases in plasma levels of IL-6 at 6 hours (data not shown) and IL-10 at 3 and 6 hours, as compared with controls ($p<0.05$). Pretreatment with those inhibitors did not show significant prevention from AP-increased plasma levels of IL-6 (data not shown). Pretreatment with aprotinin, pefabloc or trypsin inhibitor significantly prevented from AP-increased plasma levels of IL-10 at 3 hours (Fig. 1A; $p<0.05$) and pefabloc and trypsin inhibitor at 6 hours, as compared with AP animals pretreated with saline (Fig. 1B; $p<0.05$).

MPO levels in the pancreas significantly increased 3 and 6 hours after induction of pancreatitis, as compared to controls ($p<0.05$), which was significantly prevented by the pretreatment with aprotinin, pefabloc or trypsin inhibitor at 3 hours (Fig. 2A; $p<0.05$), and with aprotinin, trypsin inhibitor, polymyxin B or staurosporine at 6 hours (Fig. 2B; $p<0.05$). MPO levels in the lungs in AP significantly increased as compared to controls at 3 hours ($p<0.05$). Pretreatment with pefabloc, trypsin inhibitor or polymyxin B significantly decreased MPO levels, as compared with the AP and saline group at 3 hours (Fig. 3; $p<0.05$), but not at 6 hours (data not shown).

Protease activity in the pancreas significantly increased 3 and 6 hours after induction of AP, as compared to controls ($p<0.01$), while pretreatment with aprotinin, pefabloc or staurosporine significantly prevented protease activity as compared with AP and saline group at 3 hours (Fig. 4A; $p<0.05$). AP-induced protease activity in the pancreas significantly decreased following the administration of aprotinin and staurosporine at 6 hours (Fig. 4B; $p<0.05$).

The catalytic activity of PLA_2 in ascites significantly increased 3 and 6 hours after induction of pancreatitis, as compared with controls ($p<0.01$). Pretreatment with aprotinin, pefabloc or trypsin inhibitor significantly prevented from AP-increased PLA_2 activity in ascites at 3 hours, as compared with AP and saline group (Fig. 5A; $p<0.05$). PLA_2 activity in ascites significantly decreased following the administration of aprotinin or trypsin inhibitor at 6 hours (Fig. 5B; $p<0.01$).

Discussion

An imbalance between the pro-and anti-inflammatory response leads to localized tissue destruction and distant organ injury (Makhija and Kingsnorth, 2002). The elevated IL-6 levels in AP may serve as markers of severity of pancreatitis (Galloway and Kingsnorth, 1994), as well as being associated with the occurrence of remote organ complications (Suzuki et al., 2000). Plasma IL-10 levels have been reported to correlate with the severity of pancreatitis and could also be used as an indicator of severity (Chen et al., 1999). The magnitude of the anti-inflammatory response in patients with AP have been demonstrated to predict the clinical outcome (Mentula et al., 2004). Administration of IL-10 diminished lung injury and mortality in experimental pancreatitis (Osman et al., 1998; Rongione et al., 1997). In the present study, we found that the increased plasma concentrations of IL-6 did not significantly decrease following pretreatment with protease and PKC inhibitors. However, the elevated concentrations of IL-10 in plasma can be prevented after administration of protease inhibitors. Inhibition of proteases in AP in vivo decreased plasma levels of the anti-inflammatory cytokine IL-10, but not PKC inhibitors. We also observed the lack of inhibitory effect by aprotinin on plasma IL-10 levels at 6 hours as compared to pefabloc and trypsin inhibitor. This might be due to the integrated effect from both protease inhibition and anti-inflammation (Waxler and Rabito, 2003). It is suggested that PKC may not be involved in the pathways that are inducing IL-10 production, as detected in plasma. Due to the complexity of the cytokine activity and interactions in a setting of severe tissue injury, it is though important to further clarify the biological role of IL-6 and IL-10.

The sequestration of inflammatory cells, particularly neutrophils, within the pancreas is generally believed to be an early and important event in the evolution of both pancreatitis and pancreatitis-associated remote organ dysfunction (Poch et al., 1999). Neutrophil depletion partially reduces pancreatic damage in some models of AP but offers almost complete protection against pancreatitis-associated lung injury (Bhatia et al., 1998; Inoue et al., 1995). Pancreatic MPO levels serve as an indicator of neutrophil sequestration in the pancreas (Song et al., 2002) and similarly concerning lung injury (Lundberg et al., 2000; Song et al., 2002). In our study, MPO levels in the pancreas significantly decreased after administration of protease and PKC inhibitors, indicating a prevention against neutrophil infiltration into the pancreas and consequently a decrease in pancreatic injury. A loss of preventive effect by pefabloc pretreatment at 6 hours compared to aprotinin could be explained by the fact that aprotinin has the effect on inflammation (Waxler and Rabito, 2003). This might indicate inhibitive effect of both proteases and inflammation could last longer than simple protease inhibition. During neutrophil infiltration within the pancreas, proteases might represent earlier occurring regulatory factors than the PKC signaling pathway. It is also possible that the duration of the effects of PKC inhibitors may be longer in the pancreas or at least long enough to involve intracellular signaling transduction from the cell membrane to the nucleus through NF-kB. The exact pharmacokinetics of these inhibitors though remain to be clarified. AP-associated lung injury, implied by increased MPO levels in the lungs, decreased after pretreatment with protease and PKC inhibitor. Thus, the neutrophil infiltration into the lungs could be mediated by both PKC and proteases. Different function between polymyxin B and staurosporine may result from relative selective specificity of polymyxin B, since different function of subunits of PKC and different inhibition of other kinases were

noted (Hu, 1996; Gordge and Ryves, 1994; Wood and Osborne, 1997). We also observed different effects in MPO levels in the pancreas and the lungs following administration of these inhibitors. One possible explanation could be different pharmaceutical reactions between the primary compromised organ (pancreas) and secondary compromised organs (like the lungs) in experimental AP. Taken together, inhibition of PKC and proteases could ameliorate pancreatic and pulmonary injury.

Activated pancreatic proteases may exert digestive and thereby harmful effects on the pancreatic tissue, an important role in AP (Hartwig et al., 1999; Singh et al., 2001). We noticed that pretreatment with PKC and protease inhibitors significantly prevented against an otherwise occurring increase in pancreatic protease activity in animals with AP. Of the three protease inhibitors used, aprotinin demonstrated a long-term effect on inhibition of proteases, probably due to its anti-inflammatory properties, while the lack of inhibitory effect by the trypsin inhibitor could be that inhibition of trypsin alone is not enough for total protease inhibition in the pancreas. Interestingly, the PKC inhibitor staurosporine could also prevent agaainst an increase in protease activation, indicating that the PKC signaling pathway may also be involved in the activation of pancreatic proteases. PKC activation may take part in the pancreatic injury in AP by inhibiting acinar secretion after reorganization of the actin cytoskeleton (Siegmund et al., 2004). It is also possible that PKC-δ, a PKC isoform, regulates pancreatic amylase release by affecting the late phase of secretion (Li et al., 2004). Various PKC inhibitors, including staurosporine, H-7 and bisindolylmaleimide, have been shown inhibit amylase release (Ederveen et al., 1990; Verme et al., 1989). It seems that PKC is associated with pancreatic acinar injury via the regulation of acinar secretion.

Activation of phospholipase A_2 contributes to the pancreatic damage and systemic complications during AP (Friess et al., 2001). The PLA_2 inhibitor was able to protect the pancreas against tissue damage (Uhl et al., 1998). Phospholipase A_2 mainly catalyze the hydrolysis of the sn-2 fatty acyl chain of several phospholipid substrates to yield fatty acids and phospholipids, which are important substrates for prostaglandin, prostacyclin, thromboxane, and leukotriene synthesis, potent inflammatory mediators contributing to cell injury (Yedgar et al., 2000). PKC can result in the activation of PLA_2 (Chakraborti, 2003.), while PKC is activated by a variety of proteases (Chakraborti et al., 2004; Hashimoto and Yamamura, 1989). Inhibition of PKC and proteases involved in the activation of PLA_2 during cellular injury points at key events that can be used to prevent cellular injury. In the present study, pretreatment with protease inhibitors, rather than PKC inhibitors, significantly decreased PLA_2 activity in ascites. Aprotinin, as compared to pefabloc, provided long-term protease inhibition and anti-inflammatory effects. These findings suggested that the PKC signaling pathway might contribute to the initiation of PLA_2 activation. An increase in (Ca_2^+)i and activation of mitogen-activated protein kinase (MAPKs) is also required for PLA_2 activation and translocation from cytosol to the membrane, except for PKC activation (Chakraborti, 2003). Other pathways may contribute to the inhibiton of the catalytic activity provided by PLA_2 in ascites and e.g. a close relationship between protease activity and the corresponding (Ca_2^+)i release from stores within the same subcellular compartment exists (Kruger et al., 2000).

In conclusion, aprotinin induces major protection in the pancreas without affecting the production of IL-10. Sequestration of neutrophils to the pancreas and lungs may be regulated by PKC and proteases. The inhibition of proteases and the PKC signaling

pathway could ameliorate the increase in the pancreatic protease activity after induction of AP. Pretreatment with PKC inhibitors and protease inhibitors may provide a potential therapeutic effect in AP, though further studies have to investigate this. The inhibitory effect on both proteases and inflammation, however, lasts longer than single protease inhibition and could provide a part in multimodal treatment in AP.

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LEGENDS TO FIGURES

Fig. 1. Plasma levels of IL-10 as measured 3 hours (A) and 6 hours (B) after induction of acute pancreatitis (AP) and controls. + and * stand for $p<0.05$ as compared to controls and AP and saline pre-treatment, respectively.

Fig. 2. Myeloperoxidase (MPO) levels in the pancreas as measured 3 hours (A) and 6 hours (B) after induction of acute pancreatitis (AP) and controls. + and * stand for $p<0.05$ as compared to controls and AP and saline pre-treatment, respectively.

Fig. 3. Myeloperoxidase (MPO) levels in the lungs as measured 3 hours after induction of acute pancreatitis (AP) and controls. + and * stand for $p<0.05$ as compared to controls and AP and saline pre-treatment, respectively.

Fig. 4. Protease activity in the pancreas as measured 3 hours (A) and 6 hours (B) after induction of acute pancreatitis (AP) and controls. + + stands for $p<0.01$, * stands for $p<0.05$ as compared to controls and AP and saline pre-treatment, respectively.

Fig. 5. PLA_2 activity in ascites as measured 3 hours (A) and 6 hours (B) after induction of acute pancreatitis (AP) and controls. + + stands for $p<0.01$, * and ** stand for $p<0.05$ and $p<0.01$ as compared to controls and AP and saline pre-treatment, respectively.

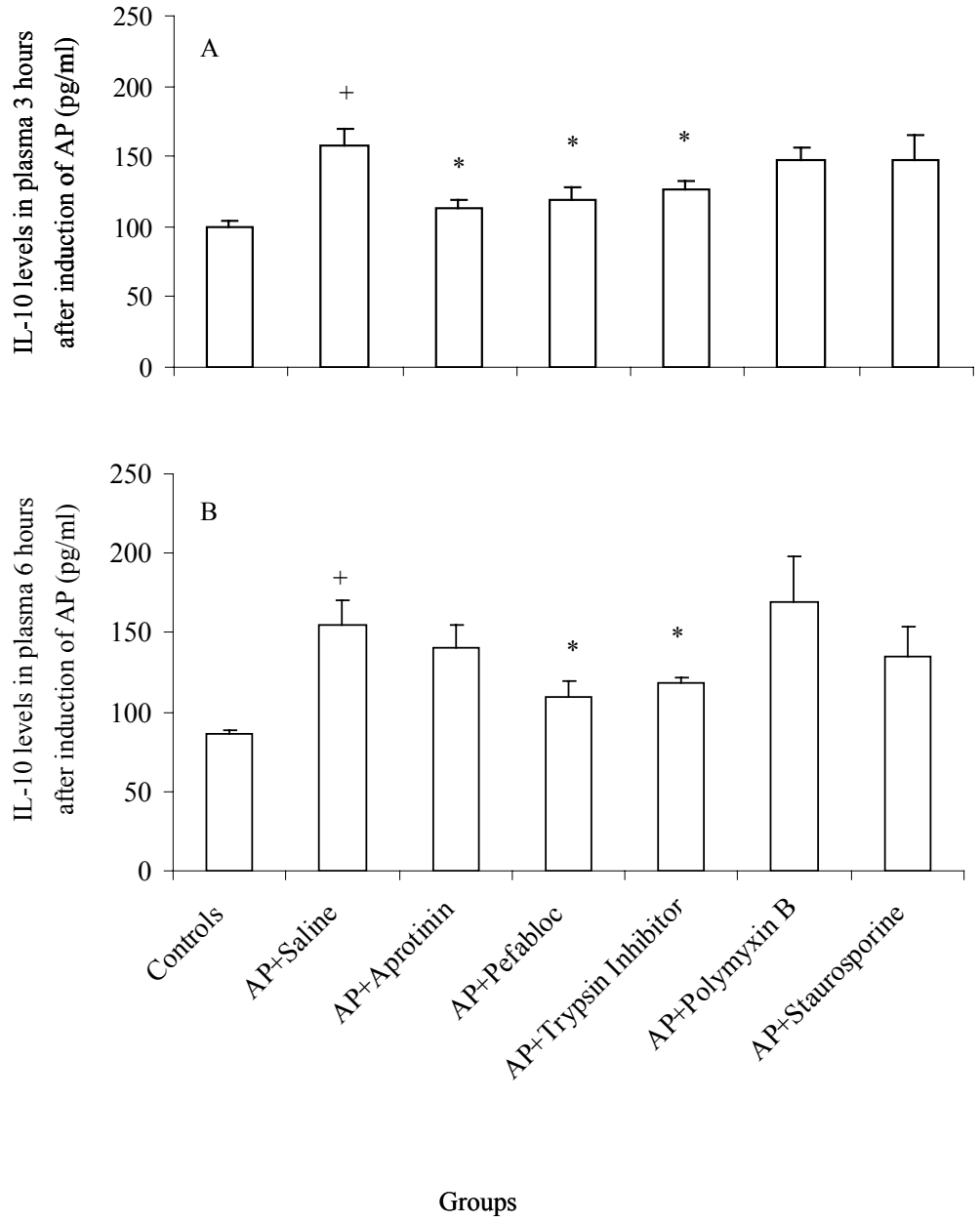
A
B
IL-10 levels in plasma 3 hours after induction of AP (pg/ml)
IL-10 levels in plasma 6 hours after induction of AP (pg/ml)
250
200
150
100
50
0
+
*
Controls
AP+Saline
AP+Aprotinin
AP+Pefabloc
AP+Trypsin Inhibitor
AP+Polymyxin B
AP+Staurosporine
Groups

Fig. 1

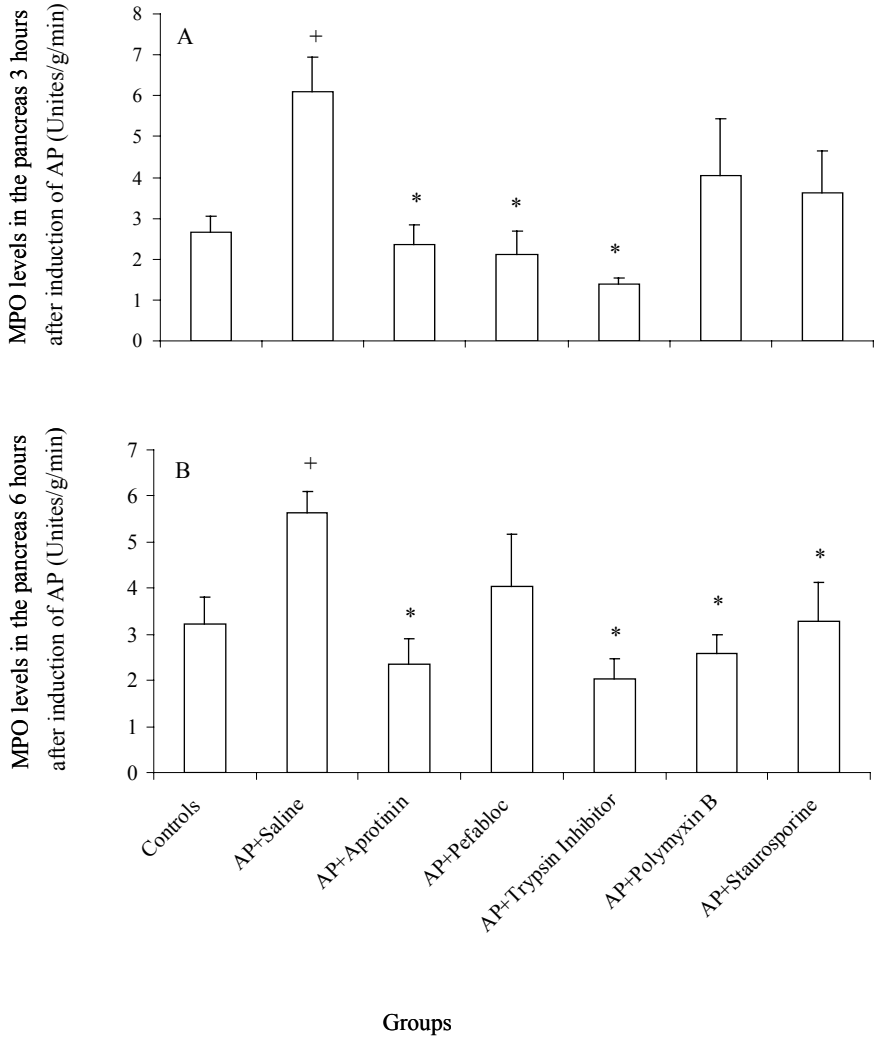
MPO levels in the pancreas 3 hours after induction of AP (Unites/g/min)
A
MPO levels in the pancreas 6 hours after induction of AP (Unites/g/min)
B
Controls
AP+Saline
AP+Aprotinin
AP+Pefabloc
AP+Trypsin Inhibitor
AP+Polymyxin B
AP+Staurosporine
Groups

Fig. 2

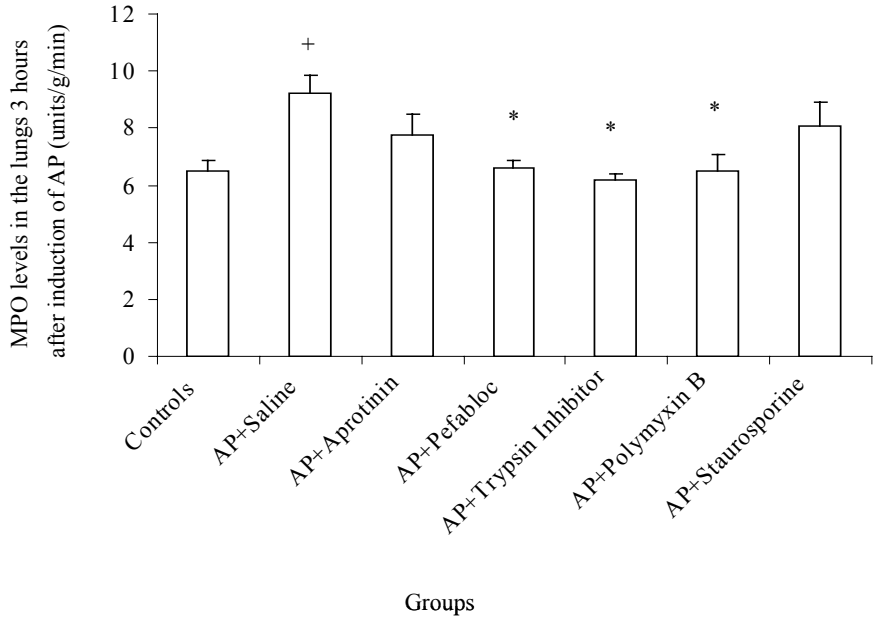
MPO levels in the lungs 3 hours after induction of AP (units/g/min)
12
10
8
6
4
2
0
+
*
*
*
Controls
AP+Saline
AP+Aprotinin
AP+Pefabloc
AP+Trypsin Inhibitor
AP+Polymyxin B
AP+Staurosporine
Groups

Fig. 3

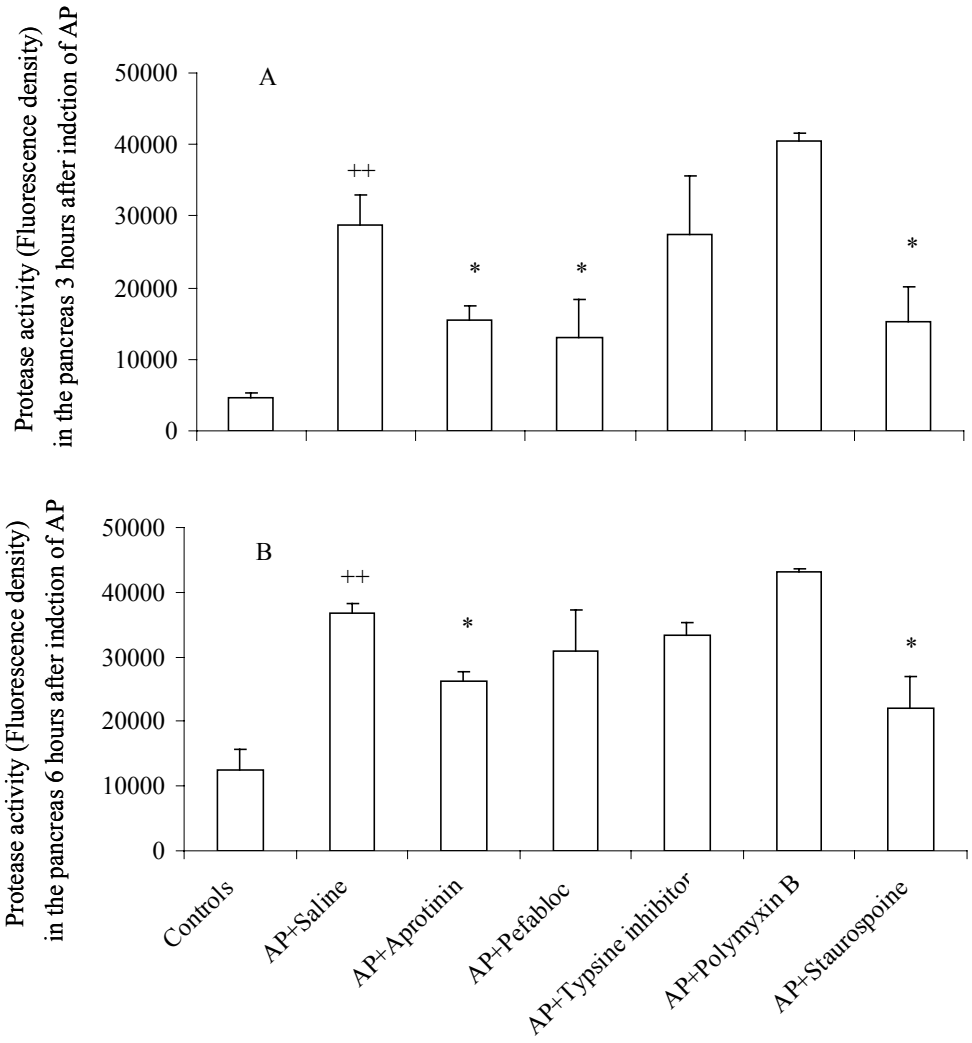
Protease activity (Fluorescence density) in the pancreas 3 hours after indction of AP
A
50000
40000
30000
20000
10000
0
++
*
*
*
Protease activity (Fluorescence density) in the pancreas 6 hours after indction of AP
B
50000
40000
30000
20000
10000
0
++
*
*
Controls
AP+Saline
AP+Aprotinin
AP+Pefabloc
AP+Typsine inhibitor
AP+Polymyxin B
AP+Staurospoine
Groups

Fig. 4

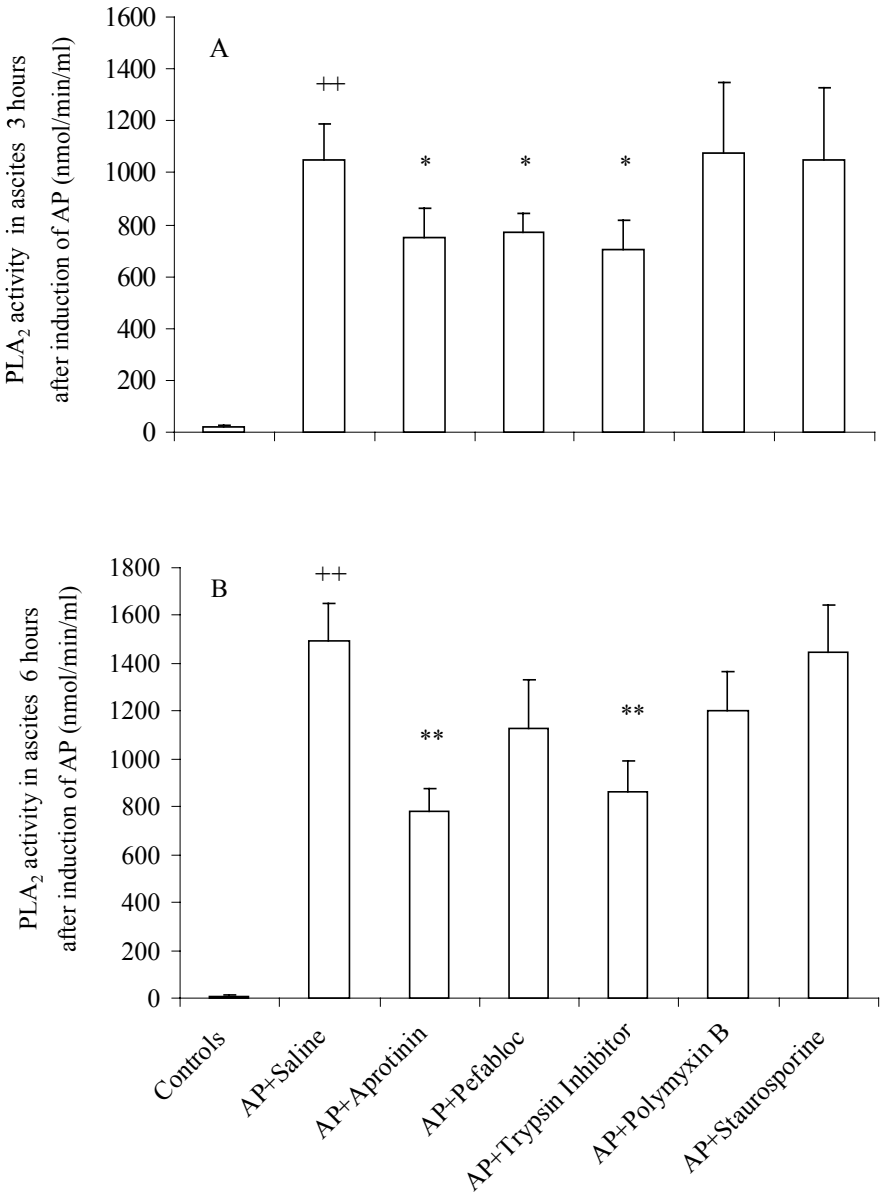
A
PLA2 activity in ascites 3 hours after induction of AP (nmol/min/ml)
1600
1400
1200
1000
800
600
400
200
0
++
*
*
*
B
PLA2 activity in ascites 6 hours after induction of AP (nmol/min/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
++
**
**
Controls
AP+Saline
AP+Aprotinin
AP+Pefabloc
AP+Trypsin Inhibitor
AP+Polymyxin B
AP+Staurosporine
Groups

Fig. 5