AAS 38/III
Recent Progress on Kinins

EFFECTS OF PROTEASE INHIBITORS IN EXPERIMENTAL SEPTIC SHOCK

Siebeck M, Spannagl M, Hoffmann H, Fink E

Departments of Surgery, Medicine, Clinical Chemistry and Clinical Biochemistry
University of Munich, Klinikum Innenstadt, Munich, Germany

SUMMARY: Endotoxin shock and contact system activation were used to study effects of hirudin, antithrombin III, eglin C, aprotinin, and [Arg15]-aprotinin in anesthetized pigs. Alterations in the systemic and pulmonary circulation were in part prevented by administration of the inhibitors.

INTRODUCTION

The proteolytic cascade systems of plasma are activated both in patients with septic shock and in animals with endotoxin-induced shock. In severe inflammation, digestive proteases are released from inflammatory cells such as phagocytes. In order to study potential therapeutic applications of various protease inhibitors we investigated their effect in experimental shock, induced by bacterial lipopolysaccharide (LPS) or dextran sulfate (DXS), an agent which activates the contact system of blood coagulation.

MATERIALS AND METHODS

We used miniature pigs (body weight ≈20 kg) under general anesthesia and controlled ventilation. All animal procedures were approved by the Regierung von Oberbayern and complied with the Tierschutzgesetz in der Fassung vom 18. 8. 1986. We infused LPS from *S. abortus equi* for 6 h or for 8 h, or DXS in a dose of 5 mg/kg in 1 h or saline. We used the following proteinase inhibitors, each in randomized, controlled trials: a purified human antithrombin III-heparin complex (AT III-heparin) or recombinant hirudin for thrombin; recombinant eglin C for PMN elastase; bovine aprotinin or recombinant [Arg15]-aprotinin for plasma kallikrein. Case numbers were mentioned in the legends. For full experimental details, the reader is referred to the original descriptions (**1**, **2**, **3**, **4**). [Arg15]-aprotinin was given intravenously as a bolus before and as a continuous infusion during stimulation with dextran sulfate. The study design and plasma levels thus achieved are given in table 1.

Table 1. Dextran sulfate (DXS) and [Arg15]-aprotinin: Doses and plasma concentrations

Group	n	Activation with	[Arg15]-aprotinin Bolus Injection (mg)	Continuous Infusion (mg)	Plasma Level at 1 h (μM)
NaCl Control	3	NaCl	0	0	0
A15A Control	2	NaCl	120	240	3.8
DXS + 360 mg	5	DXS	120	240	1.4
DXS + 180 mg	5	DXS	60	120	0.8
DXS + 90 mg	5	DXS	30	60	0.7
DXS + 45 mg	5	DXS	15	30	0.2
DXS Control	5	DXS	0	0	0

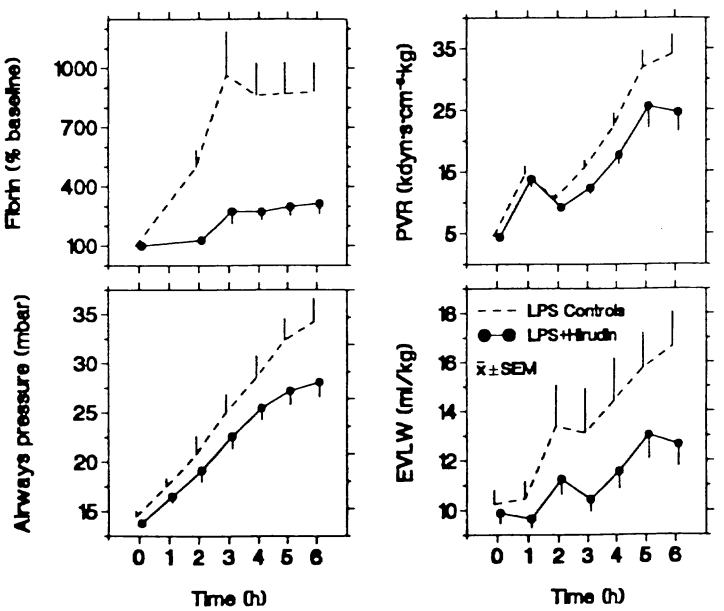

Figure 1. Effect of hirudin in LPS shock. Upper left: Soluble fibrin concentration in plasma. Upper right: Pulmonary vascular resistance (PVR). Lower left: Peak airways opening pressure. Lower right: Extravascular lung water (EVLW). All four parameters were lower in hirudin-treated animals (n=18) than in control animals (n=18).

The total intravascular protein content was calculated as the product of plasma volume as assessed by Evans' Blue dye dilution times total protein concentration in plasma (Biuret reaction), corrected for body weight. The difference between measurements obtained at baseline and after 4 h represents the loss of intravascular protein. Cardiac output and extravascular lung water were measured by single indicator thermodilution (Model 9310, Edwards), the pulmonary vascular pressure with a Swan-Ganz catheter, and the systemic blood pressure with a catheter in the abdominal aorta.

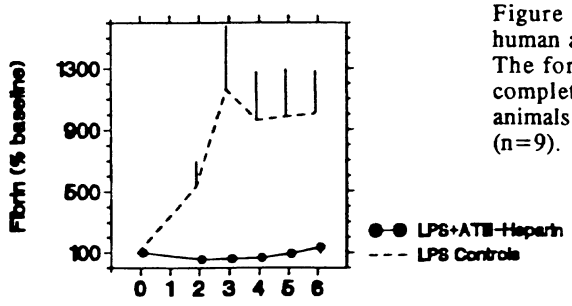

Figure 2. Effect of a purified complex between human antithrombin III and heparin in LPS shock. The formation of monomeric fibrin was almost completely blocked in the AT III-heparin-treated animals (n=8) as compared to the control animals (n=9).

Kinin-containing kininogen was determined on the basis of the amount of kinin releasable in plasma samples by incubation with trypsin. C3a in plasma was measured by radioimmunoassay. In the hirudin and AT III-heparin studies, soluble fibrin was detected using a functional assay (5). In the aprotinin study, fibrin monomer was detected in plasma by an enzyme immunoassay using a monoclonal antibody to the N-terminal α-chain of fibrin.

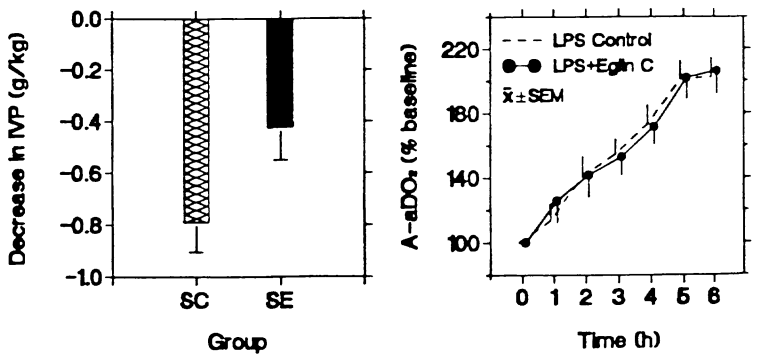

Figure 3. Effect of eglin C in LPS shock. Left: Decrease in intravascular protein content (IVP). Eglin-treated animals (n=18) had substantially less decrease in IVP than control animals (n=18). Right: Alveolar-arterial O_2 pressure gradient (A-aDO_2). There was no difference in A-aDO_2 between controls and eglin-treated animals.

RESULTS

Hirudin (0.1 μM plasma concentration) reduced fibrin formation and ameliorated LPS-induced lung dysfunction (cf. fig. 1). AT III-heparin (120% of normal AT III activity in plasma) blocked fibrin formation (cf. fig. 2), but did not improve respiratory dysfunction. Eglin C (2 μM) reduced the loss of protein from the intravascular space, but did not affect respiratory dysfunction (cf. fig. 3).

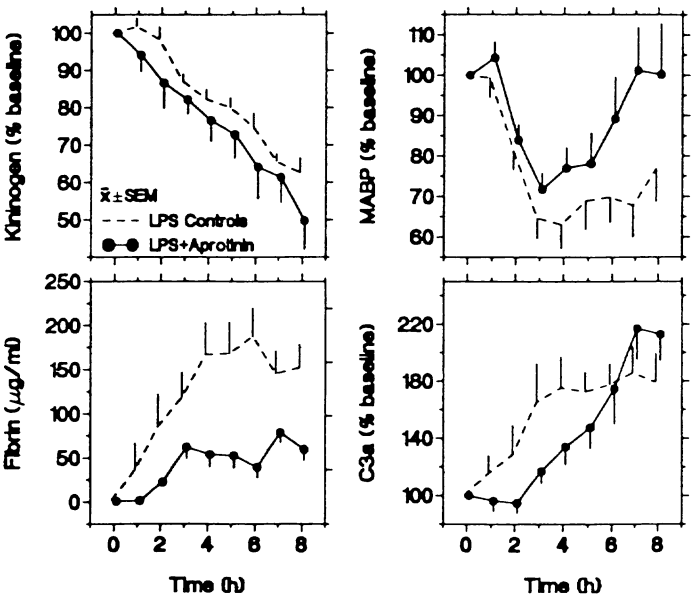

Figure 4. Effect of aprotinin in LPS shock. Upper left: Kinin-containing total kininogen. There was no difference in turn-over of kininogen between aprotinin-treated animals (n=10) and control animals (n=10). Upper right: Mean arterial blood pressure (MABP). Aprotinin-treated animals had higher blood pressure than control animals. Lower left: Monomeric fibrin concentration in plasma. Lower right: Complement fragment 3a (C3a) concentration in plasma. Aprotinin-treated animals had substantially lower fibrin levels and, at least initially, lower C3a levels than control animals.

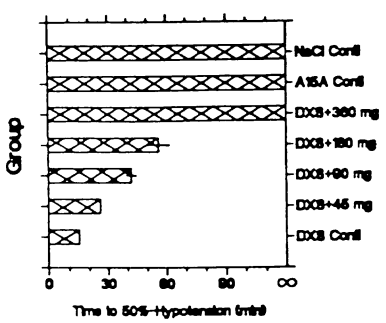

Figure 5. Effect of [Arg15]-aprotinin in dextran sulfate (DXS)-induced hypotension. Time from onset of DXS infusion to 50% arterial hypotension was progressively delayed with increased doses of [Arg15]-aprotinin; hypotension was completely abolished in the highest dose administered.

Aprotinin (10 µM) reduced LPS-induced hypotension, fibrin formation and C3a formation, but not the decrease in plasma kininogen levels (cf. fig. 4). [Arg15]-aprotinin (1.4 µM) blocked DXS-induced arterial hypotension (cf. fig. 5) and kininogen breakdown.

DISCUSSION

Hirudin had the anticipated effect on fibrinogen degradation and fibrin formation. Furthermore, it reduced some of the effects of LPS on lung function. In contrast, antithrombin III in complex with heparin had no effect on respiratory dysfunction, although it was even better at inhibiting fibrinogen degradation and completely blocked fibrin formation. Studies with heparin alone in a similar model revealed analogous results (**6**). Although these studies were not designed specifically to compare the effectiveness of hirudin and antithrombin III, we assume that hirudin in contrast to antithrombin III was able to block some of the cellular effects of thrombin (mediated by formation of thromboxane (**7**) or endothelin (**8**) or directly (**9**)), and this is a possible reason why hirudin ameliorated LPS-induced lung dysfunction and reduced the increase in pulmonary vascular resistance.

Eglin C was found only to have an effect in the systemic circulation where it reduced protein extravasation. Similar effects have been observed in two other independent experiments (**10, 11**). Three possible explanations exist for this finding. PMN elastase can influence the metabolism of arachidonic acid (**12**). Chymase, another enzyme that is inhibited by eglin C, is involved in mast cell degranulation, and it is possible that the observed effects of eglin C have nothing to do with PMN elastase, but rather with inhibition of mast cell degranulation (**13**). The third possibility is an action mediated *via* cytokines. Human alpha-1 antitrypsin (**14**) and, similarly, eglin C (A. Wendel, Konstanz, FRG, personal communication) are able to block a final proteolytic step in the generation of bioactive tumor necrosis factor α. The absence of an effect of eglin C on respiratory dysfunction suggests that the endotoxin-induced lung damage in our model was produced by other mechanisms than the release of PMN elastase.

Aprotinin produced effects that suggest that the contact system of plasma was activated in LPS shock. Hypotension and vasodilation are typical effects of bradykinin. Complement activation is associated with activation of the contact system. The observed effect on the C3a plasma levels however might also have been produced by plasmin inhibition, and the aprotinin plasma levels in our experiment were far higher than what is necessary to achieve plasmin inhibition. Significant was the finding that fibrin formation, assessed by an immunologic method, was reduced – a fact that underscores the importance of this pathway for coagulation activation in LPS shock.

[Arg15]-aprotinin is an aprotinin homologue with excellent inhibition of plasma kallikrein (**15**). In our *in vivo* model using infusion of dextran sulfate it blocked at lower concentrations than aprotinin (**16**). It should be useful to test whether it has additional protective effects in LPS shock.

CONCLUSION

We have shown that different proteases are involved in different ways in the events during endotoxin shock, and that the effect of proteinase inhibition largely depends on the enzyme being inhibited and on the relative role of the enzyme in this very complex sequence of events. The impact of enzymes on organ dysfunction in shock, however, may depend on their ability to stimulate cells, in addition to their cleaving of substrates.

ACKNOWLEDGEMENT

This work was supported by the Deutsche Forschungsgemeinschaft, the Bundesinnenministerium and the Bundesministerium für Forschung und Technologie. Compounds were generously made available by C. Galanos, Freiburg, Bayer AG, Wuppertal, Plantorgan KG, Bad Zwischenahn, Ciba-Geigy AG, Basel, Switzerland, and Immuno AG, Vienna, Austria. Substantial experimental contributions were made by M. Weis, C. Keser, J. Bichler, J. Kohl, J. Weipert, P. Kroworsch, P. Scheuber, S. Moravec, M. Jochum, A. Braune, A. Oettl, and G. Godec. The authors wish to thank L. Schweiberer, W. Schramm, and H. Fritz for generous support and valuable discussion.

REFERENCES

1. Hoffmann H, Siebeck M, Spannagl M, Weis M, Geiger R, Jochum M, Fritz H (1990). Effect of recombinant hirudin, a specific inhibitor of thrombin, on endotoxin-induced intravascular coagulation and acute lung injury in pigs. Am Rev Respir Dis 142:782-788.

2. Spannagl M, Hoffmann H, Siebeck M, Weipert J, Schwarz HP, Schramm W (1991). A purified antithrombin III - heparin complex as a potent inhibitor of thrombin in porcine endotoxin shock. Thromb Res 61:1-10.

3. Siebeck M, Hoffmann H, Weipert J, Fritz H (1992). Effect of the elastase inhibitor eglin C in porcine endotoxin shock. Circ Shock 36:3, in press.

4. Siebeck M, Fink E, Weipert J, Jochum M, Fritz H, Spannagl M, Kroworsch P, Shimamoto K, Schweiberer L. Inhibition of plasma kallikrein with aprotinin in porcine endotoxin shock. J. Trauma, submitted.

5. Wiman B, Rånby M (1986). Determination of soluble fibrin in plasma by a rapid and quantitative spectrophotometric assay. Thromb Haemostas 55:189-193.

6. Griffin MP, Gore DC, Zwischenberger JB, Lobe TE, Hall M, Traber DL, Herndon DN. Does heparin improve survival in experimental porcine Gram-negative septic shock? Circ Shock 1990, 31:343-349.

7. Seeger W, Neuhof H, Hall J, Roka L (1988). Pulmonary vasoconstrictor response to soluble fibrin in isolated lungs: possible role of thromboxane generation. Circ Res 62:651-659.

8. Boulanger CM, Lüscher TF (1991). Hirudin and nitrates inhibit the thrombin-induced release of endothelin from the intact porcine aorta. Circ Res 68:1768-1772.

9. Sonne O (1988). The specific binding of thrombin to human polymorphonuclear leukocytes. Scand J Clin Lab Invest 48:831-838.

10. Hock CE, Lefer AM (1985). Beneficial effects of a neutral protease inhibitor in traumatic shock. Pharmacol Res Comm 17: 217-226.

11. Siebeck M, Hoffmann H, Jochum M, Fritz H (1989). Inhibition of proteinases with recombinant eglin C during experimental *Escherichia coli* septicemia in the pig. Eur Surg Res 21:11-17.

12. LeRoy EC, Ager A, Gordon JL (1984). Effects of neutrophil elastase and other proteases on porcine aortic endothelial prostaglandin I_2 production, adenine nucleotide release, and responses to vasoactive agents. J Clin Invest 74:1003-1010.

13. Fink E, Nettelbeck R, Fritz H (1986). Inhibiton of mast cell chymase by eglin c and antileukoprotease (HUSI-I). Biol Chem Hoppe-Seyler 367: 567-571.

14. Niehörster M, Tiegs G, Schade UF, Wendel A (1990). In vivo evidence for protease-catalysed mechanism providing bioactive tumor necrosis factor alpha. Biochem Pharmacol 40:1601-1603.

15. Auerswald EA, Hörlein D, Reinhardt G, Schröder W, Schnabel E (1988). Expression, isolation and characterization of recombinant [Arg^{15},Glu^{52}]aprotinin. Biol Chem Hoppe-Seyler 369 (Suppl):27-35.

16. Hoffmann H, Siebeck M, Thetter O, Jochum M, Fritz H (1989). Aprotinin concentrations effective for the inhibition of tissue kallikrein and plasma kallikrein *in vitro* and *in vivo*. Adv Exp Med Biol 247B:35-42.