KININS V

Part B

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APROTININ CONCENTRATIONS EFFECTIVE FOR THE INHIBITION OF TISSUE KALLIKREIN AND PLASMA KALLIKREIN IN VITRO AND IN VIVO

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INTRODUCTION

The basic proteinase inhibitor aprotinin (Trasylol^{R}) from bovine organs has been used for the treatment of acute pancreatitis, hyperfibrinolysis, and traumatic shock for more than 30 years. However, uncertainty has existed as to the appropriate dosage of aprotinin until recently, when more precise assays to monitor plasma aprotinin levels were developed (Jochum et al., 1984; Mueller-Esterl et al., 1984). These assays have been utilized to monitor aprotinin plasma concentrations in several clinical trials (Jochum et al., 1987; Clasen et al., 1987).

Plasma concentrations of aprotinin sufficient to inhibit the different target enzymes were calculated from enzyme kinetics and substrate availability (Philipp, 1978; Fritz, 1978, 1985). To experimentally assess these theoretical considerations we studied the plasma concentrations of aprotinin preventing the proteinase action of plasma kallikrein and tissue kallikrein on their natural target substrates, the high molecular weight (HMW) and low molecular weight (LMW) kininogen as follows: (i) in in vitro systems the purified components were applied (tissue kallikrein or plasma kallikrein and LMW or HMW kininogen); (ii) in experimental animal models either the purified proteinase (tissue kallikrein) was administered by infusion or the proteinase (plasma kallikrein) was liberated from its precursor by the induction of contact phase activation.

MATERIALS AND METHODS

Reagents: Aprotinin (Trasylol^{R}) was purchased as a sterile isotonic solution for intravenous administration (vials of 500,000 KIU/50 ml, pH 5, specific activity 7150 $\pm$ 200 KIU/mg from Bayer AG, Leverkusen, FRG). Human high molecular weight kininogen (HMWK), low molecular weight kininogen (LMWK) and plasma kallikrein (HPK) were isolated from freshly drawn plasma from healthy donors by the method of Mueller-Esterl et al., 1983. Tissue kallikrein from porcine pancreas (PZ 744997) was kindly donated by Bayer AG, Leverkusen, FRG. Dextran sulphate (DS) (M_r 500,000) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Units and assays. The kallikrein inactivator unit (KIU) is used to measure the activity of aprotinin. One KIU is defined as the amount of aprotinin which decreases the activity of 2 biological kallikrein units by 50 %. Multiplication of the KIU values by a factor of 0.14 transforms these figures into the corresponding microgram quantities of aprotinin. Aprotinin levels in plasma were measured by a competitive enzyme-linked immunosorbent assay (ELISA) for aprotinin (Mueller-Esterl et al., 1984). Plasma prekallikrein (PPK) levels were measured by a chromogenic substrate assay (S-2302, Kabi Vitrum). Kininogen (HMWK + LMWK) plasma levels were measured by a bradykinin radioimmunoassay after cleavage with trypsin (Uchida and Katori, 1979). Coagulation times were determined by routine methods using diagnostic kits obtained from Behring, FRG. Data are presented as mean ± SEM.

In vitro studies. All in vitro experiments using purified components were performed in phosphate buffered saline with 1 % bovine serum albumin (pH 7.2, 37°C). By incubating increasing amounts of the inhibitor with a constant amount of the proteinase and kininogen the inhibition stoichiometry of human plasma kallikrein and porcine tissue kallikrein with aprotinin was determined. After a 180 min incubation time the reaction was stopped by acidification and the kinin released was measured by kinin RIA. The concentrations of HMWK (0.8×10^{-6} mol/l) and LMWK (1.8×10^{-6} mol/l) used in vitro were similar to the corresponding physiological plasma levels.

Animal experiments. Weaned piglets weighing 17-23 kg were used. The animals were purchased from the Versuchsgut Oberschleissheim, Veterinary School, University of Munich, FRG. Acepromacine maleate 50 mg (Vetranquil^{R}) was administered intramuscularly for premedication. Anesthesia was induced with 15 mg/kg pentobarbital (Narcoren^{R}) and maintained with repeated injections of 4 mg/kg. One arterial and two venous catheters were inserted via the left femoral vessels for hemodynamic monitoring and blood sampling. After a 1-h baseline period the animals were randomly assigned to different experimental protocols (Table 1). Mean arterial blood pressure (MAP) was monitored with a Bentley Trantec Model 800 transducer and a Sirecust 404 monitor (Siemens AG, Munich, FRG). Blood samples were taken before and at 15 min intervals during the experiment. Platelets were counted manually in EDTA anticoagulated samples (Neubauer chamber). Blood samples were anticoagulated with 3.8 % citrate (1:10) for PPK, aprotinin, Quick, or aPTT determinations, or heparin (5 U/ml) for kininogen measurements, and centrifuged at 20°C with 3000 rpm for 20 min. The supernatant was aliquoted and stored at -80°C until measurement. After the observation period of 2 hours (DS groups, Table 1) and 5 hours (TK groups, Table 1), the animals were sacrificed.

Table 1. Experimental protocols: Animals were given either tissue kallikrein (TK) or dextran sulphate (DS) simultaneously with either saline or different doses of aprotinin.

Contact activation [DS 2 mg/kg]	+ saline	(n= 5)	group DS_0
	+ aprotinin 50,000 KIU/kg	(n= 4)	group DS_1
	+ aprotinin 200,000 KIU/kg	(n= 4)	group DS_2
	+ aprotinin 400,000 KIU/kg	(n= 2)	group DS_3
Tissue Kallikrein [TK 50 µg/(kg x h)]	+ saline	(n= 7)	group TK_0
	+ aprotinin 694 KIU/(kg x h)	(n= 6)	group TK_1
	+ aprotinin 1389 KIU/(kg x h)	(n= 6)	group TK_2

In animals assigned to DS groups (see Table 1) activation of the coagulation, fibrinolytic and plasma kallikrein systems was induced by continuous intravenous infusion of dextran sulphate (DS), 2 mg/kg over 60 min. In addition, five animals (group DS_0) were given saline; ten animals received aprotinin in a total dose ranging between 51,000 and 415,000 KIU/kg (groups DS_{1-3}) intravenously in two equivalent doses, one as a bolus injection before starting the DS infusion and the other dose was coadministered with DS over a 60 min period.

Tissue kallikrein (TK) was infused in 19 animals (see Table 1) at a constant rate of 50 µg/(kg x h) over 3 hours (groups TK_{0-2}). The control animals (group TK_0) received TK and saline. In the remaining animals TK was given simultaneously with a 4 hr infusion of aprotinin. The infusion of aprotinin was started 1 h before TK administration.

RESULTS

Inhibition of tissue kallikrein

Incubation of porcine tissue kallikrein with human LMW and HMW kininogen in vitro resulted in kinin release at tissue kallikrein concentrations greater than 10^{-10} mol/l. The kinin release from LMWK was effectively blocked by aprotinin levels greater than 10^{-7} mol/l (4 KIU/ml) (Fig. 1). This was also demonstrated for HMW kininogen (data not shown).

After tissue kallikrein infusion, mean arterial pressure (MAP) decreased over a 30 min period from 86.5 ± 10.3 mmHg to 76 ± 12.5 mmHg in group TK_0. After 1 h MAP was restored to baseline level. A similar transient decrease in MAP (from 100.3 ± 13.2 to 83.7 ± 24.7 mmHg) was observed in group TK_1, whereas in group TK_2 MAP decreased only slightly (from 92.6 ± 16.2 mmHg to 86.2 ± 5.1 mmHg).

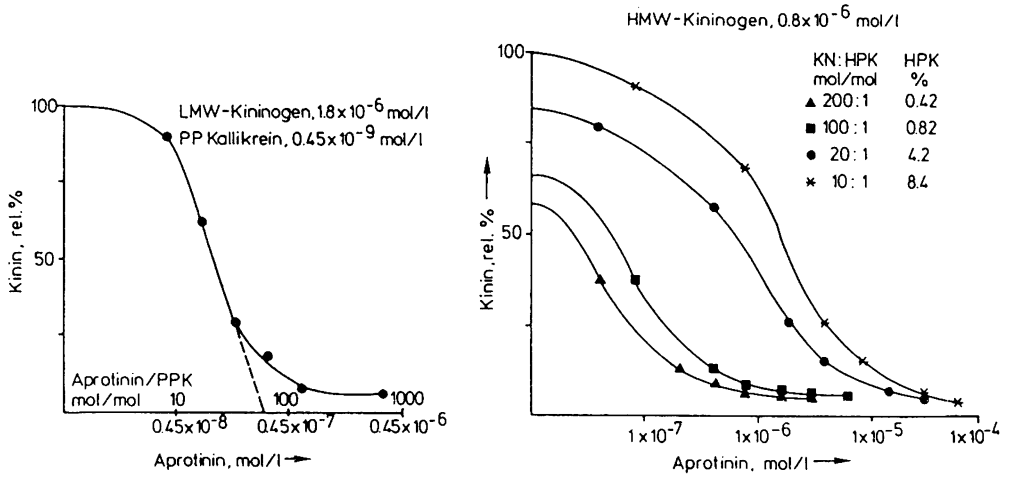

Fig. 1. Dose-response curves of aprotinin on the inhibition of kinin release from (left) human low molecular weight (LMW) kininogen by porcine pancreatic (=tissue) kallikrein (PPK) and (right) human high molecular weight (HMW) kininogen by human plasma kallikrein (HPK) in the in vitro system. The percent values given correspond to the degree of plasma prokallikrein activation in relation to the mean total amount of prokallikrein present in human plasma.

Kininogen concentrations in plasma decreased to 42 ± 17.5 % of the starting value (100 %) in group TK_0. Aprotinin administration at doses of 694 KIU/(kg x h) in group TK_1, and 1389 KIU/(kg x h) in group TK_2 limited this fall to 63 ± 6.5 %, and 85 ± 7.6 %, respectively (Fig. 2). In these experiments the plasma levels of aprotinin ranged from 0.6 to 1.4 KIU/ml in group TK_1, and from 4 to 7 KIU/ml in group TK_2.

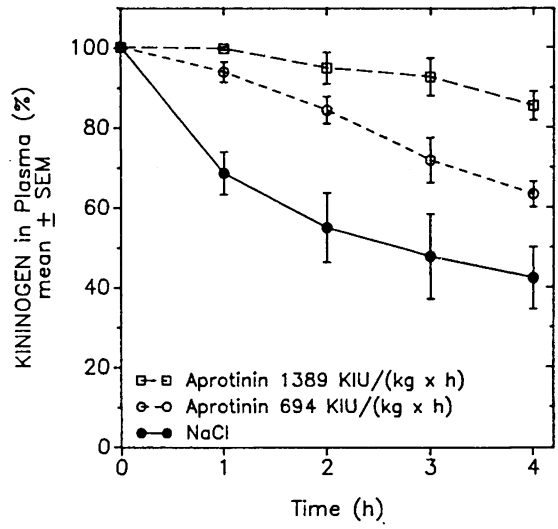

Fig. 2. Plasma kininogen concentrations in animals during an infusion of tissue kallikrein [50 µg/(kg x h)]. Aprotinin plasma levels ranged from 0.6 to 1.4 KIU/ml and from 4 to 7 KIU/ml during a continuous infusion of 694 KIU/(kg x h) and 1389 KIU/(kg x h), respectively.

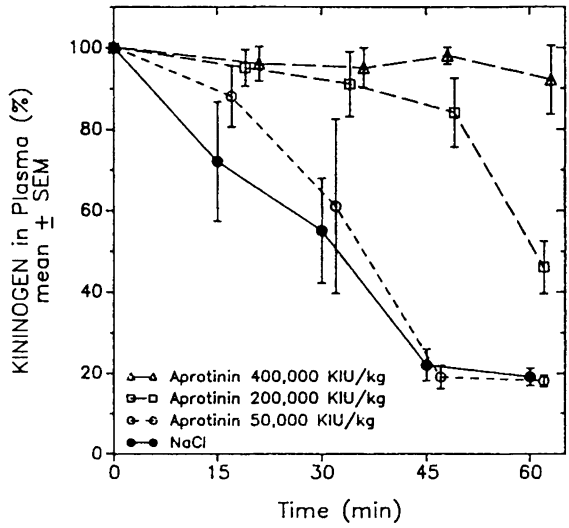

Fig. 3. Plasma kininogen concentrations in animals during activation of the intrinsic coagulation cascade by continuous infusion of dextran sulphate (DS), 2 mg/kg over 1 h. Aprotinin plasma levels ranged from 100 to 150 KIU/ml at a total dose of 50,000 KIU/kg, from 200 to 300 KIU/ml (dose: 200,000 KIU/kg), and from 400 to 500 KIU/ml at a total dose of 400,000 KIU/kg.

Inhibition of plasma kallikrein

When human plasma kallikrein (HPK) was incubated with human kininogen in the in vitro system, HPK concentrations greater than 10^{-9} mol/l were associated with substantial kinin release from HMW kininogen. HPK released kinin not only from HMWK but also from LMWK. At HPK concentrations of 10^{-8} mol/l, 40 % of total HMWK and 20 % of total LMWK was cleaved within 180 min. Aprotinin levels greater than 10^{-5} mol/l (400 KIU/ml) effectively blocked the action of HPK on HMWK (Fig.1) and on LMWK (data not shown). The actual concentrations of aprotinin necessary for complete inhibition of kinin release depended clearly on the amount of plasma kallikrein present. Thus, the inhibitory effectiveness of a certain aprotinin concentration in vivo would depend on the percentage of plasma prokallikrein activated under the given conditions.

Induction of contact activation in animal experiments by continuous infusion of dextran sulphate (DS) over 60 min led to a transient drop in MAP (-66 ± 14 mmHg) and a decrease in plasma prokallikrein (-77 ± 2 %) and kininogen levels (-81 ± 5 %). Administration of aprotinin delayed the onset of MAP reduction and the consumption of kininogen in a dose-dependent manner (administered dose vs. onset of MAP decrease: Pearson r^2=0.86; n=8). No MAP reduction at all and only a slight consumption of kininogen (-8 ± 12 %) was observed at plasma aprotinin levels of 400 - 500 KIU/ml in group DS_3 (Fig. 3). Plasma levels of aprotinin in group DS_1 ranged from 100 - 150 KIU/ml and in group DS_2 from 200 - 300 KIU/ml. Platelet count dropped below 200,000 $1/mm^3$ in 4 out of the 5 animals in group DS_0. In the aprotinin treated animals thrombocytopenia developed in 1 of the 4 animals in group DS_2 and in none of two animals in group DS_3.

DISCUSSION

The primary target enzymes of the basic proteinase inhibitor aprotinin (Trasylol^R) from bovine tissue cells, are serine proteases such as trypsin, plasmin, as well as tissue and plasma kallikrein (Fritz and Wunderer, 1983). In this study aprotinin was assayed for its ability to inhibit the action of tissue kallikrein and plasma kallikrein on their natural substrates, HMW and LMW kininogen, both in vitro and in vivo. The results demonstrate that effective inhibition of tissue kallikrein and plasma kallikrein may be achieved, in vitro and in vivo under concentration conditions known to occur clinically. Human tissue kallikrein and human plasma kallikrein have affinities to aprotinin comparable to those of the corresponding enzymes of porcine origin (Fritz and Wunderer, 1983). Our in vitro studies using purified human compounds, and the in vivo studies using pigs, yielded comparable results. Therefore the inhibitory plasma levels determined may be equally valid in humans. Plasma levels of aprotinin were measured by a recently developed competitive enzyme-linked immunosorbent assay (Mueller-Esterl et al., 1984). This assay has been shown to provide a versatile means to monitor rapidly and precisely aprotinin levels in plasma and body fluids from patients treated with aprotinin (Jochum and Mueller-Esterl, 1985; Clasen et al., 1987).

The activation and release of pancreatic proteases such as trypsin or tissue kallikrein into peritoneal exudates and into the circulation, concomitant with the consumption of endogenous inhibitors and the turnover of kininogen, has been demonstrated in both experimental pancreatitis (Kortmann et al., 1983; Borgstrom and Ohlsson, 1978) as well as clinically in man (Balldin and Ohlsson, 1979). Using continuous tissue kallikrein infusion as a model for kallikrein liberation, e.g. during acute pancreatitis, we studied the inhibition of tissue kallikrein with aprotinin in animal experiments. The dose of kallikrein infused was calculated from the kallikrein

amount present in the porcine pancreas (Frey et al., 1968). The results obtained revealed that even the maximal continuously liberated dose of kallikrein, as can be predicted in vivo, may be sufficiently blocked at aprotinin plasma levels of 4 - 10 KIU/ml (10^{-7} mol/l). This was assessed by kininogen consumption and measurements of arterial pressure. Keeping in mind that trypsin is even more sensitive to aprotinin inhibition, concentrations of 10^{-7} mol/l should also suffice to completely inactivate trypsin released from the pancreas under inflammatory conditions (Fritz, 1985).

Unlike tissue kallikrein and trypsin, plasma kallikrein (HPK) is much less efficiently inhibited by aprotinin (Fritz, 1978). HPK plays a major role in the activation of the coagulation and fibrinolytic cascades. (i) It triggers the endogenous pathway of blood coagulation via F XII activation, (ii) it liberates kinin from kininogen, and (iii) it may stimulate polymorphonuclear granulocytes and thus induce the release of lysosomal enzymes (Mueller-Esterl and Fritz, 1984; Schapira et al., 1983). A pronounced and rapid activation of HPK has been demonstrated in traumatized and septic patients, and during cardiopulmonary bypass (McConn et al., 1983; Aasen, 1985; Heller et al., 1987). Activation of HPK occurs on negatively charged surfaces, e.g. exposed subendothelial structures. Also the cell wall fractions of bacteria may activate HPK (Kalter et al., 1983). Thus, after trauma and sepsis, in a short time period, high amounts of active HPK may be generated from its precursor plasma prokallikrein despite high concentrations of endogenous HPK inhibitors present in plasma.

We used the intravenous infusion of dextran sulphate (DS) as a model for the induction of in vivo contact activation. In this model about 80 % of the plasma prokallikrein is activated within 60 minutes. This activation is paralleled by a transient fall in arterial pressure, a rapid turnover of the kininogens, a fall in platelet count, and a prolonged coagulation time. Aprotinin was able to attenuate this response to DS in a clear dose dependend manner. Marginal inhibition was observed at plasma levels of 100 KIU/ml, weak inhibition with 200 KIU/ml, while plasma levels greater than 400 KIU/ml abolished the effect completely. This was confirmed in the in vitro studies: When physiological concentrations of HMWK were incubated with small amounts of HPK (corresponding to 1 % of the total HPK pool activatable in vivo) kinin release was inhibited at aprotinin concentrations of 40 KIU/ml, whereas when higher amounts of HPK were applied (corresponding to 10-20 % of the total HPK pool), only aprotinin concentrations as 400 KIU/ml properly inhibited the action of HPK on kininogen. These results are in agreement with theoretical considerations based on enzyme kinetics and substrate availability which predicted that only aprotinin concentrations of 200-400 KIU/ml would suffice to effectively inhibit plasma kallikrein (Fritz, 1978; Philipp, 1978).

At exceedingly high aprotinin plasma levels the inhibitory function of aprotinin may be explained in part by alternative mechanisms. At pH 7.4 aprotinin is a highly positive charged molecule and may avidly bind to negatively charged surfaces (Fritz and Wunderer, 1983). In our model, aprotinin may directly bind to DS and thus saturate the negatively charged activating surface. In addition, it is known from ACD blood that aprotinin (at concentrations of 400 KIU/ml) may bind to negatively charged molecules of the platelet membrane surface and thus inhibit platelet aggregation (Harke et al., 1982). This effect of aprotinin, based on ionic interactions may be responsible for the absence of thrombocytopenia in the high dose aprotinin group. However, this non specific effect of aprotinin, in addition to its role as an enzyme inhibitor may be significant in certain clinical situations such as after trauma or during cardiopulmonary bypass.

The question arises as to the feasibility of obtaining these plasma concentrations of aprotinin in clinical situations. Recent studies have been

shown that this is indeed feasible (Clasen et al., 1987). However, due to the short plasma half life time of the inhibitor a continuous infusion is necessary to maintain plasma levels within the desired range (Fritz et al., 1969, Clasen et al., 1987). Very high doses of aprotinin are apparently tolerated well. Coagulatory or microcirculatory disturbances have not been observed after administration of 17.5 millon KIU aprotinin within 24 hours in traumatized patients (Clasen et al., 1987). The dosage regimen used in this study resulted in initial peak plasma levels of about 400 KIU/ml which returned to 100-200 KIU/ml during subsequent continuous infusion of 1 million KIU/h over 12 hours. In our study, in two aprotinin-control animals showing peak plasma levels of 800 KIU/ml after a bolus injection and 400 to 500 KIU/ml during subsequent continuous infusion of aprotinin, apart from a slight prolongation of the coagulation time, no physiologic change was noted.

In conclusion, the present study demonstrated that effective inhibition of tissue kallikrein and plasma kallikrein in vivo is achieved at aprotinin plasma levels of 4 - 10 KIU/ml (10^{-7} mol/l) and 400 KIU/ml (10^{-5} mol/l), respectively. In conjunction with the newly developed assays for aprotinin this data may help to improve the efficacy of proteinase inhibitor therapy.

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