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EFFECTS OF A HIGHLY SELECTIVE SYNTHETIC INHIBITOR OF PLASMA KALLIKREIN ON DISSEMINATED INTRAVASCULAR COAGULATION IN RATS

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ABSTRACT We found a new, highly selective plasma kallikrein inhibitor, *trans*-4-aminomethyl-cyclohexanecarbonylphenylalanine 4-carboxymethylanilide hydrochloride, called PKSI-527 in our laboratories. This study was conducted to evaluate PKSI-527, on thromboplastin (TP)- and endotoxin (LPS)-induced disseminated intravascular coagulation (DIC) in rats. PKSI-527 was infused intravenously at 0.1 mg/kg/min for 250 min. Three of the parameters of the coagulation and fibrinolysis system, fibrinogen level, platelet counts and fibrin(ogen) degradation products (FDP) level were assayed. PKSI-527 prevented the change in the coagulation and fibrinolysis system in LPS-induced DIC, however it was not clearly effective in TP-induced DIC. The parameters of organ failure, such as serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), creatine phosphokinase (CPK), lactate, blood urea nitrogen and β-glucuronidase, were assayed. Although the changes in the fibrinogen level, platelet counts and FDP level were almost the same in both models, the parameters of organ failure apparently increased in LPS-induced DIC more so than in TP-induced DIC. PKSI-527 significantly suppressed the increases in GOT and GPT in LPS-induced DIC. These results indicate that plasma kallikrein may play a significant role in LPS-induced DIC. Therefore, PKSI-527, as a synthetic plasma kallikrein inhibitor may be a valuable tool to explore the mechanism of DIC and the accompanying organ failure.

Although disseminated intravascular coagulation (DIC) has been a major clinicopathologic and therapeutic challenge over the past 20 years, the treatment of DIC still remains a problem because DIC develops as a complication of many different disorders, and may have a variety of clinical manifestations. Moreover, DIC is often associated with multiple organ failure (MOF), especially in sepsis, which is a major cause of death and disability among hospitalized patients (1-3).

On the other hand, in patients with DIC in sepsis, decreased levels of plasma factor XII, prekallikrein and kallikrein inhibitor are seen (4,5), which indicates that the activation of the contact system may be involved in the progression of DIC and MOF.

Keywords: Disseminated intravascular coagulation, synthetic protease inhibitor, plasma kallikrein, multiple organ failure.
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Plasma prekallikrein is a factor in the contact system, and becomes activated through the activation of factor XII by contact-activation. Plasma kallikrein (PK) is known to participate in the activation of blood coagulation and fibrinolysis as well as in kinin generation, as seen by analysis of Fletcher trait plasma and so on (6-8). Furthermore, Egberg et al. suggested that PK infusions into pigs activated the intrinsic coagulation system and the fibrinolysis system, which are signs of DIC (9).

Recently, we were able to find a highly selective inhibitor of PK, PKSI-527, which inhibits the coagulation, fibrinolysis and kinin generation induced by contact activation *in vitro* (10-12). In the present study , we evaluated the effect of PKSI-527 on two DIC models, TP-induced DIC and LPS-induced DIC, and considered the possible role of the PK in DIC and MOF.

MATERIALS AND METHODS

1. Animals

Male Sprague-Dawley rats (Charles River Japan, 210-240 g) were used in this study. They were housed in a temperature- and light-controlled room and provided with laboratory rodent chow and water *ad libitum*. The animals were fasted overnight before the experiment.

2. Test Reagents

Prior to use, LPS (lipopolysaccharide B, *Escherichia.coli* 0127; B8; Difco Laboratories, USA) was dissolved in physiological saline, and thromboplastin (TP, Simplastin® ; Organon Teknika Co., Belgium) was dissolved in distilled water. PKSI-527 *trans*-4-aminomethylcyclohexanecarbonyl-phenylalanine 4-carboxymethylanilide hydrochloride was synthesized in our laboratory.

3. Animal Experiments

The rats were divided into three groups: saline, DIC-control and DIC-PKSI treatment groups. Rats in the DIC-PKSI treatment group were anesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg) and DIC was induced by intravenous administration of either TP or LPS. TP (20 mg/kg/4 h) was infused via the femoral vein, and LPS (10 mg/kg) was injected via the tail vein. PKSI-527, dissolved in a 5% glucose solution, was infused intravenously via the femoral vein for a total of 250 min, starting 10 min before the induction of DIC. Rats in the DIC-control and saline groups were submitted to the same procedure, except that those in the DIC-control group were given 5% glucose solution instead of PKSI-527, and those in the saline group were given 5% glucose solution (instead of PKSI-527 and TP or LPS). After 4 h, blood was withdrawn from the abdominal aorta and used for determination of platelet count (collected with EDTA), fibrinogen (collected with 3.8% sodium citrate), fibrin(ogen) degradation products (FDP, in serum with Trasylol ®) and other laboratory values (collected without anticoagulant). At the same time, urine samples were withdrawn from the bladder for *N*-acetylglucosamine (NAG) determination.

4. Blood and Urine Analysis

i) Parameters of blood coagulation and fibrinolysis: The platelet count was determined using a hemoanalyzer (Sysmex-CC 180A, Toa Medical Electronics, Kobe, Japan). Fibrinogen concentrations were determined using the Ratnoff-Menzie method (13). FDP concentrations were determined using the staphylococcal clumping method (14) (Sigma Chemical, St. Louis, USA) and expressed as a proportion of the highest dilution of serum displaying the clumping phenomenon.

ii) Parameters of organ failure: The following parameters were determined using commercially available diagnostic kits - GOT and GPT (Transaminase CII-Test Wako® ,Wako Pure Chemical Industries, Japan); blood urea nitrogen (BUN, Monotest Urea® , Boehringer Mannheim, Germany); creatine phosphokinase (CPK, Sigma Chemical); and NAG (Meiassay NAG® , Meiji Seika, Japan).

iii) Parameters of shock: β-glucuronidase (Sigma Chemical) and lactate (Boehringer Mannheim) were also determined using commercially available diagnostic kits.

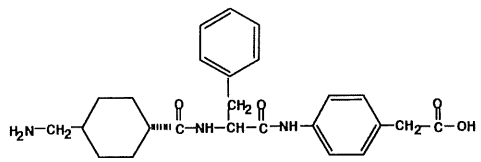
5. Statistical Analysis

All data are expressed as mean ± SEM, except where otherwise noted. The groups were compared using Scheffe's methods and for FDP levels, the Kruskal-Wallis test was performed for significant p values of 5% or less were regarded as significant.

RESULTS

Characteristics of PKSI-527

Figure 1 shows the chemical structure and enzyme inhibition of PKSI-527. PKSI-527 is a derivative of phenylalanine, which has a simple three-component structure. The N- and C-terminals are replaced with *trans*-4-aminomethylcyclohexanecarboxylic acid (*t*-AMCHA) and *p*-carboxy-methylaniline, respectively. PKSI-527 inhibits PK with a K_i value of 0.81 μM, and the K_i values of this substance with glandular kallikrein, plasmin, urokinase, factor Xa and thrombin, are more than 200 times greater. The LD_{50} in mice is more than 150 mg/kg IV. These results indicate that PKSI-527 is a potent, highly selective, and relatively non-toxic inhibitor of PK.



Enzyme	PL	P.Kall	G.Kall	Th	FXa	UK
Ki(μM)	390	0.81	>500	>500	>500	200

FIG.1
Chemical structure and enzyme-inhibitory effects of PKSI-527.

Comparison between TP- and LPS-induced DIC

First, the doses of TP and LPS were adjusted so that they produced nearly equivalent changes in platelet count, fibrinogen level and FDP level in the rats. It was found that infusion of 20 mg/kg/4 h of TP (n=11) had approximately the same effect on these parameters as 10 mg/kg of LPS (n=11), that is, the platelet counts were $43.4 \pm 5 \times 10^4$ and $45 \pm 5 \times 10^4/\mu l$, the fibrinogen levels were 71 ± 28 mg/dl and 44 ± 6 mg/dl, and the highest FDP dilution ratios were 160-2560 and 320-2560, in the TP- and LPS-treated groups, respectively. These values all differed significantly from those in the normal group (Fig. 2).

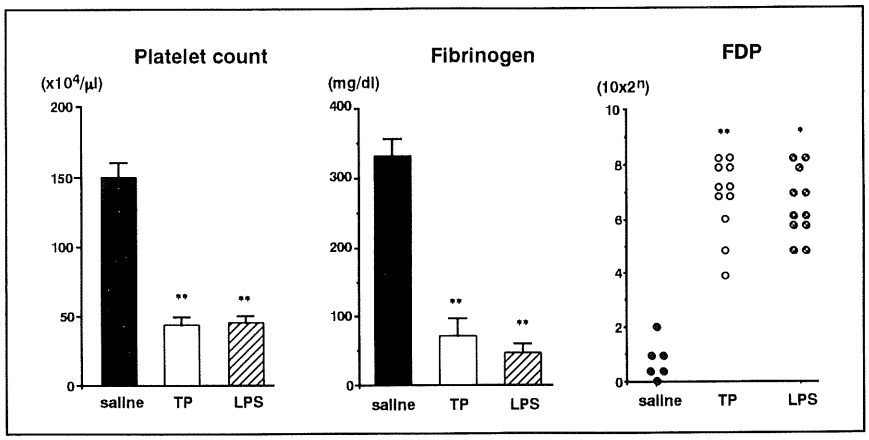


FIG.2

Comparison of TP- and LPS-induced DIC with regard to blood coagulation and fibrinolysis (platelet count, fibrinogen level, and FDP level). Values are expressed as mean ± SEM. The FDP level indicates the highest dilution ratio of serum for each animal. (Details in Materials and Methods) * $p<0.05$, ** $p<0.01$ for DIC-control group vs. saline group. There were no significant differences between TP-induced and LPS-induced DIC.

Using these doses, various parameters of organ failure and shock were determined in both models (Fig. 3). The GOT, GPT, CPK, BUN, β-glucuronidase and lactate levels were 183±21 U/ml, 23.3±7.0 U/ml, 533±67 mU/ml, 24.7±2.2 mg/dl, 31.3±3.3 U/ml and 17.0±1.5 mg/dl, respectively in TP-induced DIC, and 554±75 U/ml, 160±53 U/ml, 883±125 mU/ml, 37.0±1.9 mg/dl, 46.7±5.0 U/ml and 22.0±1.7 mg/ml, respectively, in LPS-induced DIC. These values were significantly higher in LPS-induced DIC than in TP-induced DIC.

Effect of PKSI-527

In LPS-induced DIC, the administration of PKSI-527 (0.1 mg/kg/min for 250 min) significantly suppressed the decrease in platelet count and fibrinogen level as well as the increase in FDP level which usually occur in DIC (Fig.4) , especially the increases in GOT and GPT levels (Fig. 5); however, PKSI-527 had no significant effect on the concentrations of CPK, NAG, BUN or β-glucuronidase (Table 1).

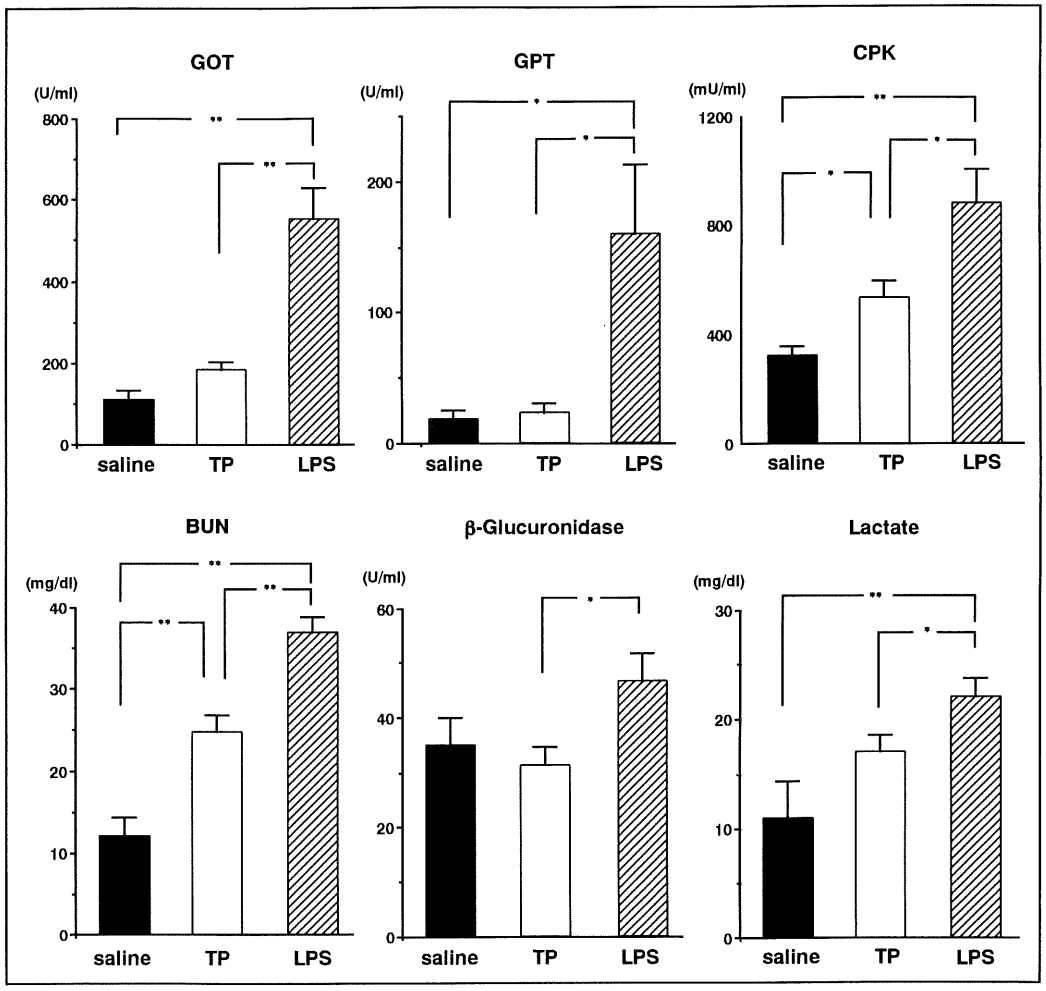


FIG. 3

Comparison of TP- and LPS-induced DIC with regard to organ dysfunction (GOT, GPT, BUN, CPK) and shock (β-glucuronidase, lactate) markers. *$p<0.05$ **$p<0.01$ for DIC-control group vs. saline group.

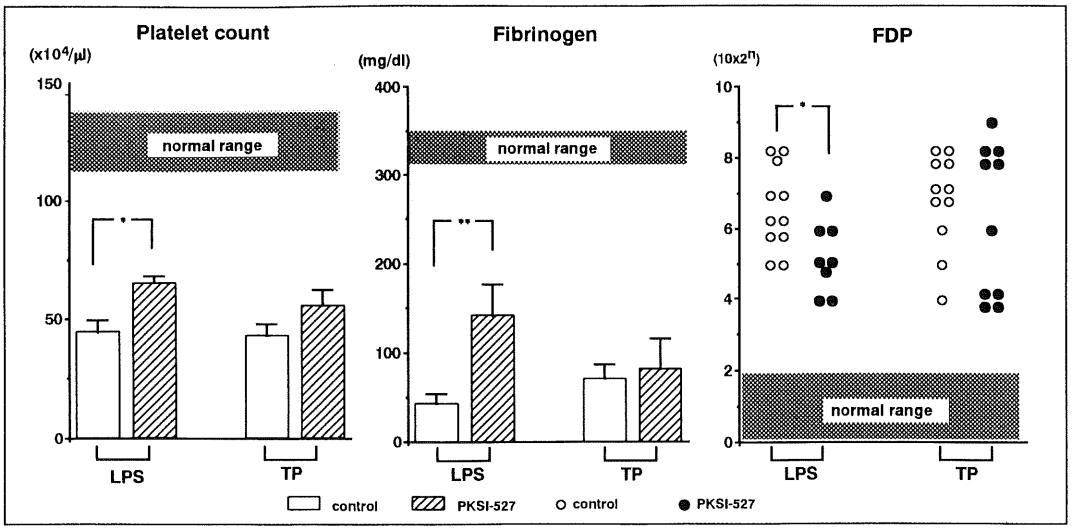


FIG. 4

Effect of plasma kallikrein inhibitor (PKSI-527) on TP- and LPS-induced DIC with regard to changes in parameters of blood coagulation and fibrinolysis (platelet count, fibrinogen level, FDP level). PKSI-527 was intravenously infused at the dose of 0.1 mg/kg/min for 250 min. *$p<0.05$ **$p<0.01$ for the comparison of the PKSI-527 treated group with the DIC-control group.

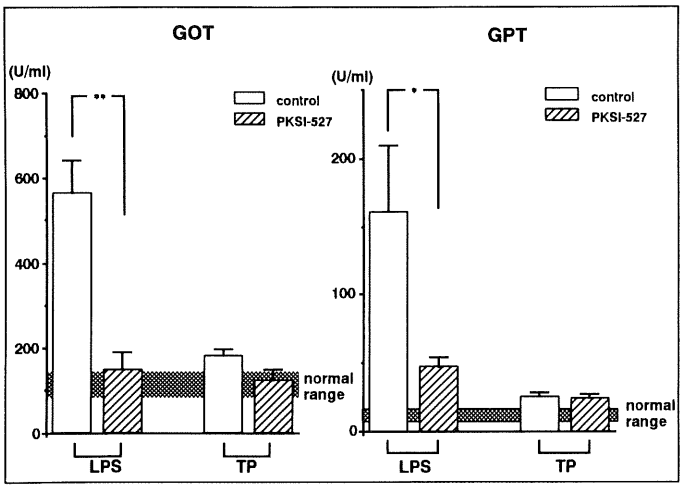


FIG.5

Effect of plasma kallikrein inhibitor (PKSI-527) on LPS- and TP-induced DIC with regard to changes in of GOT and GPT. *$p<0.05$, **$p<0.01$ for the comparison of the PKSI-527 treated group with the DIC-control group.

TABLE I

Effects of PKSI-527 on CPK, NAG, BUN, β-Glucuronidase in LPS-induced DIC.

	CPK mU/ml	NAG U/ml	BUN mg/dl	β-glucuronidase U/ml
control	883±103	127±13	37±2	46±4
PKSI-527	618±112	89±14	35±2	57±6

DISCUSSION

The contact system consists of four factors, factor XII, factor XI, high-molecular-weight kininogen and plasma prekallikrein, and it is known to closely participate in the initiation of coagulation, fibrinolysis and the kinin system. In patients with DIC, especially in sepsis, these contact factors are consumed, suggesting that the contact system is involved in the development of DIC (4, 5).

Recently, we were able to find a newly selective synthetic inhibitor of PK, PKSI-527 (10, 12). The dissociation constant (K_i value) of PKSI-527 for PK is 0.81 μM, and K_i values for glandular kallikrein, plasmin, thrombin, urokinase and factor Xa are more than 200 times greater, which indicates extremely high selectivity of PKSI-527 for PK. PKSI-527 inhibits the coagulation, fibrinolysis and kinin generation induced by contact activation in human plasma *in vitro* (11). In the present study, we evaluated the effect of PKSI-527 on two DIC models, TP-induced DIC and LPS-induced DIC, and considered the possible role of PK in DIC.

First of all we compared two experimental models of DIC: LPS- and TP-induced DIC in rats. When the administration doses of LPS and TP were adjusted to produce nearly the same effects on fibrinogen, FDP levels and platelet count, it was found that the parameters of organ failure and shock (GOT, GPT, CPK, BUN, β-glucuronidase and lactate) were significantly higher in the LPS-treated rats than in the TP-treated rats (Fig. 3). These results suggest that LPS-induced DIC is easily to accompany with MOF, and may be reflecting the aspects of clinical reports that a high incidence of MOF complication with DIC due to sepsis or infection (1-3).

The administration of PKSI-527 suppressed the changes in platelet count, fibrinogen and FDP levels in LPS-induced (but not TP-induced) DIC, suggesting the participation of PK in coagulation and fibrinolysis induced by only LPS (Fig. 4). Egberg et al. reported that infusion of PK to pigs activated the coagulation and fibrinolysis system, and it was shown that signs of DIC could be induced by PK. DaLa Cadena et al. reported that intravenous administration of endotoxin in humans activated the kallikrein-kinin system (15). Moson et al. and Lämmle et al. reported that contact factors were consumed in patients with DIC due to endothelial injury or endotoxemia, but not in DIC associated with leukemia, carcinoma, or abortion (4,5). These reports and our data suggest that the contact system is involved in DIC due to sepsis, the involvement of which might vary with the underlying disorders. On the other hand, Müller-Berghaus and Schneberger reported that factor XII is not the first coagulation factor activated after the intravenous injection of endotoxin into rabbits (16). Pixlay et al. also reported that monoclonal antibody to factor XII did not suppress the decrease of fibrinogen level, factor V level and platelet count in *E. coli* injected-balloon sepsis model, and the decrease of these factors was suppressed by monoclonal antibody to tissue factor (17, 18). They concluded that activation of the contact system might not be the most important initiator of DIC and that this role might be played by tissue factor. It is known that the contents of contact factors differ greatly among species (19, 20), and that endotoxin can liberate tissue factor from endothelial cells and macrophage (21, 22). Therefore, the relationship between intrinsic and extrinsic coagulation in LPS-induced DIC, which may be greatly different on dosage of LPS or on the species of certainly animal used, is complex.

On the parameters of organ failure, PKSI-527 clearly suppressed the increases in GOT and GPT, and showed a tendency to reduce the levels of CPK or NAG, suggesting that PK is involved in organ failure. PK is known to be chemotactic to human neutrophils and monocytes, and it causes neutrophil aggregation and secretion of elastase (23-26). Ferreina et al. reported that BK stimulates the release of cytokines from macrophages (27). On the other hand, it has also been suggested that activated leukocytes play an important role in the organ damage induced by LPS, and that fibrin formation is not a major contributor to the organ damage (28-30). PKSI-527 may suppresses the action of cytokines through the inhibition of PK and thus improve MOF. However, further studies are necessary to clarify this point, and PKSI-527, could be a useful tool for this purpose. The present study suggests that PK plays a role in LPS-induced DIC. PKSI-527, as synthetic selective PK inhibitor appears to be a valuable tool in the study of the mechanism

of DIC and MOF, and might open up a new field of chemical control of organ failure.

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REFERENCES

1. FRY, D.E. Humoral mediators of MOF. Arch Surg *121*, 201-208, 1986
2. AIKAWA, N., SHINOZAWA, Y., ISHIBIKI, K. and ABE,O. Clinical analysis of multiple organ failure in burned patients. Burns *13*, 103-109, 1987
3. FRY, D.E., GARRISON, R.N., HEITSCH, R.G., CALHOUN, K. and POLK, H.C. Determinants of death in patients with intraabdominal abscess. Surgery *89*, 517-523, 1980
4. MASON, J.W. and COLMAN, R.W. The role of Hageman factor in disseminated intravascular coagulation induced by septicemia, neoplasia, or liver disease. Thromb Diathes Haemorragh *26*, 325-331, 1971
5. LÄMMLE, B., TRAN, T.H., RITZ, R. and DUCKERT, F. Plasma prekallikrein, factor XII, antithrombin III, C_1-inhibitor and α_2-macroglobulin in critically III patients with suspected disseminated intravascular coagulation (DIC). Am J Clin Pathol *82*, 396-404, 1984
6. WEISS, A.S., GALLIN, J.I. and KAPLAN, A.P. Fletcher factor deficiency. A diminished rate of Hageman factor activation caused by absence of prekallikrein with abnormalities of coagulation, fibrinolysis, chemotactic activity, and kinin generation. J Clin Invest *53*, 622-633, 1974
7. SAITO, H., RATNOFF, O.D. and DONALDSON, V.H. Defective activation of clotting, fibrinolytic, and permeability-enhancing systems in human Fletcher trait plasma. Cir Res *34*, 641-651, 1974
8. DONALDSON, V.H., SAITO, H. and RATNOFF, O.D. Defective esterase and kinin-forming activity in human Fletcher trait plasma: a fraction rich in kallikreinlike activity. Cir Res *34*, 652-662, 1974
9. EGBERG, N., GALLIMORE,M.J. and JAKOBSSON, J. Effects of plasma kallikrein infusions into pigs on haemodynamic and haemostatic variables. Fibrinolysis *2*, 95-100, 1988
10. OKAMOTO, S., OKAMOTO, U., WANAKA, K., HIJIKATA-OKUNOMIYA, A., BOHGAKI, M., NAITO, T., HORIE, N. and OKADA, Y. Highly selective synthetic inhibitors with regard to plasma-kallikrein activities. KININ V, part B, eds. Abe, K., Moriya, H. and Fujii, S. Plenum Press, New York-London, 1989. pp 29-34
11.WANAKA, K., OKAMOTO, S., BOHGAKI, M., HIJIKATA-OKUNOMIYA, A., NAITO, T. and OKADA, Y. Effect of a highly selective plasma-kallikrein synthetic inhibitor on contact activation relating to kinin generation, coagulation and fibrinolysis. Thromb Res *57*, 889-895, 1990
12. WANAKA, K., OKADA, Y., TSUDA, Y., OKAMOTO, U., HIJIKATA-OKUNOMIYA, A. and OKAMOTO, S. Synthesis of trans-4-aminomethylcyclohexanecarbonyl-L- and -D-phenylalanine-4-carboxymethylanilide and examination of their inhibitory activity against plasma kallikrein. Chem Pharm Bull *40*, 1814-1817, 1992
13. RATNOFF, O.D. and MENZIE, C. A new method for determination of fibrinogen in small samples of plasma. J Lab Clin Med *37*, 316-320, 1951
14. ALLINGTON, MJ. Fibrinogen and fibrin the degradation products and the clumping of staphylococci by serum. Brit J Haemat *13*, 550-567, 1967
15. DELA CADENA, R.A., SUFFREDINI, A.F., PAGE, J.D., PIXLEY, R.A., KAUFMAN, N., PARRILLO, J.E. and COLMAN, R.W. Activation of the kallikrein-kinin system after endotoxin administration to normal human volunteers. Blood *81*, 3313-3317,1993
16. MÜLLER-BERGHAUS, G. and SCHNEBERGER, R. Hageman factor activation in the generalized Shwartsman reaction induced by endotoxin. Br J Haematol *21*, 513-527, 1971
17. PIXLEY, R.A., DA LA CADENA, R., PAGE, J.D., KAUFMAN, N., WYSHOCK, E.G., CHANG, A., TAYLOR, F.B. Jr. and COLMAN, R.W. The contact system contributes to hypotension but not disseminated intravascular coagulation in lethal bacteremia. J Clin Invest *91*,

61-68, 1993
18. TAYLOR, F.B. Jr, CHANG, A., RUF, W., MORRISSEY, J.H., HINSHAW, L., CATLETT, R., BLICK, K. and EDGINGTON, T.S. Lethal E coli septic shock is prevented by blocking tissue factor with monoclonal antibody. Circ Shock *33*, 127-134, 1991
19. SAITO, H., GOLDSMITH, G. and RATNOFF, O.D. Fletcher factor activity in plasmas of various species. Proc Soc Exp Biol Med *147*, 519-523, 1974
20. SAITO, H., GOLDSMITH, G. and WALDMANN, R. Fitzgerald factor (high molecular weight kininogen) clotting activity in human plasma in health and disease in various animal plasmas. Blood *48*, 941-947, 1976
21. SCARPATI, E.M. and SADLER, J.E. Regulation of endothelial cell coagulant properties: Modulation of tissue factor, plasminogen activator inhibitors, and thrombomodulin by phorbol 12-myristate 13-acetate and tumor necrosis factor. J Biol Chem *264*, 20705-20713, 1989
22. GREGORY, S.A., MORRISSEY, J.H. and EDGNGTON, T.S. Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin. Mol Cell Biol *9*, 2752-2755, 1989
23. KAPLAN, A.P., KAY, A.B. and AUSTEN, K.F. A prealbumin activator of prekallikrein. III. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. J Exp Med *135*, 81-97, 1972
24. GALLIN, J.I. and KAPLAN, A.P. Mononuclear cell chemotactic activity of kallikrein and plasminogen activator and its inhibition by C_1 inhibitor and α_2-macroglobulin. J Immunol *113*, 1928-1934, 1974
25. SCHAPIRA, M., DESPLAND, E., SCOTT, C.F., BOXER, L.A. and COLMAN, R.W. Purified human plasma kallikrein aggregates human blood neutrophils. J Clin Invest *69*, 1199-1202, 1982
26.WACHTFOGEL, Y.T., KUCICH, U., JAMES, H.L., SCOTT, C.F., SCHAPIRA, M., ZIMMERMAN, M., COHEN, A.B. and COLMAN, R.W. Human plasma kallikrein releases neutrophil elastase during blood coagulation. J Clin Invest *72*, 1672-1677, 1983
27. FERREIRA, S.H., LORENZETTI, B.B., CUNHA, F.Q. and POOLE, S. Bradykinin release of TNF-α plays a key role in the development of inflammatory hyperalgesia. Agents Actions (Suppl.) *38*, C7-C9, 1993
28. TAYLOR, F.B.Jr., CHANG, A.C.K., PEER, G.T., MATHER, T., BLICK, K., CATLETT,R., LOCKHART, M.S. and ESMON,C.T. DEGR-factor Xa blocks disseminated intravascular coagulation inhibited by *Escherichia coli* without preventing shock or organ damage. Blood *78*, 364-368, 1991
29. GAYNOR, E., BOUVIER, C. and SPAET, T.H. Vascular lesions: possible pathogenetic basis of the generalized Shwartzman reaction. Science *170*, 986-988, 1970
30. UCHIBA, M., OKAJIMA, K., MURAKAMI, K., OKABE, H. and TAKATSUKI, K. Endotoxin-induced pulmonary vascular injury is mainly mediated by activated neutrophils in rats. Thromb Res *78*, 117-125, 1995