Anticoagulant Action of Vanadate

Takayuki FUNAKOSHI,[a] Hideaki SHIMADA,[a] Shoji KOJIMA,[a] Shozo SHOJI,[b] Yukiho KUBOTA,[b] Tetsuo MORITA,[c] Nobuaki TOMINAGA,[c] and Hiroshi UEKI*,[c]

Department of Hygenic Chemistry[a] and Biochemistry,[b] Faculty of Pharmaceutical Sciences, Kumamoto University, 5–1 Oehon-machi, Kumamoto 862, Japan and Department of Biochemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University,[c] 985 Higashimura-machi, Fukuyama 729–02, Japan. *Received July 1, 1991*

Sodium orthovanadate (vanadate) prolonged the clotting time of normal human plasma in a dose-dependent manner. The prolongation of clotting time by vanadate linearly decreased with an increase in the concentration of amiloride. Vanadate also was completely additive to prolongation by heparin. When factor Xa or thrombin was incubated with vanadate, the amidolytic activity of each decreased in a dose-dependent manner with vanadate. Amiloride protected the decrease of amidolytic activity of both factor Xa and thrombin by vanadate. The amidolytic activity of trypsin also was inhibited by vanadate, but that of α-chymotrypsin was not inhibited, suggesting that vanadate preferentially inhibits the amidolytic activity of trypsin and trypsin-like enzymes. These results show that vanadate prolongs the clotting time of plasma through mechanisms involving in part the inhibition of the activity of both factor Xa and thrombin.

Keywords sodium orthovanadate; anticoagulant action; factor Xa; thrombin; human plasma; amiloride; proteinase

Vanadium is an essential trace element, as confirmed by experiments employing animals fed on a vanadium-deficient diet.[1,2] Its compound, sodium orthovanadate (vanadate), mimics many effects of insulin *in vitro* and *in vivo*, including the activation of glycogen synthase, the stimulation of glucose incorporation into lipids, the suppression of hormone-dependent lipolysis, and the stimulatory phosphorylation of an insulin receptor in isolated rat adipocytes and the normalization of blood glucose levels of streptozotocin-induced diabetic rats.[3–5] We reported that vanadate stimulated the release of lipoprotein lipase (LPL) activity from isolated rat fat pads into the incubation medium through mechanisms involving amiloride-sensitive and intracellular Ca^{2+}-dependent processes with a requirement of metabolic energy.[6] Heparin also stimulates the release of LPL activity from cultured avian adipocytes and its mechanism is accounted for by an increased secretion rate and decreased degradation rate but not by an increased synthesis rate.[7] The stimulatory release by vanadate, as well as that by heparin, was not due to an increase in protein synthesis.[6] In general, physiological events that are important in controlling blood loss are thought to proceed *via* the following reactions: The adhesion of platelets and release of vasoactive amines from it, triggering of coagulation process, and activation of the fibrinolytic system.[8] Heparin is a well known potent anticoagulant that is proposed to act by activating antithrombin with respect to the neutralization of thrombin and by exerting its action through a mechanism differing from the stimulatory release of LPL activity from the fat pads.[9] Therefore, we investigated whether or not vanadate prolongs the kaolin-induced clotting time of normal human plasma.

The present paper shows that vanadate prolongs the clotting time of normal human plasma through mechanisms involving in part the inhibition of activity of both factor Xa and thrombin.

Materials and Methods

Materials The sources of chemicals used in this work were as follows: Vanadate (Na_3VO_4), heparin (167 U/mg), dibutyryl cyclic adenosine monophosphate (AMP) and 3-isobutyl-1-methylxanthine from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), genistein from K & K Laboratories (Cleveland, OH), amiloride, biochanin A, factor X and thrombin from human plasma, enzyme activating factor X from Russell's viper venom, trypsin (type XIII, from bovine pancreas), α-chymotrypsin (type VII, from bovine pancreas), and rabbit brain cephalin from Sigma Chemical Co. (St. Louis, MO), *tert*-butyloxycarbonyl (Boc)–Val–Pro–Arg–4-methyl-coumaryl-7-amide (MCA), Boc–Ile–Glu–Gly–Arg–MCA, Boc–Phe–Ser–Arg–MCA, and succinyl (Suc)–Ala–Ala–Pro–Phe–MCA from Peptide Institute Inc. (Osaka, Japan). All other chemicals used were of analytical grade.

Assay of Clotting Activity Routine assay of anticoagulant activity of vanadate was performed by kaolin-induced clotting time using rabbit brain cephalin, as described previously.[10]

Determination of Enzymatic Activity Factor X was activated by a Russell's viper venom enzyme, and factor Xa was separated by a Waters high performance liquid chromatography system equipped with a TSK 3000SW column, as described by Fujikawa *et al.*[11] The amidolytic activity of factor Xa was determined with Boc–Ile–Glu–Gly–Arg–MCA, as described previously.[10] Amidolytic activities of thrombin, trypsin, and α-chymotrypsin were determined with Boc–Val–Pro–Arg–MCA,[12] Boc–Phe–Ser–Arg–MCA,[13] and Suc–Ala–Ala–Pro–Phe–MCA,[14] respectively.

Results and Discussion

Figure 1 shows the anticoagulant action of vanadate on normal human plasma. Kaolin-induced clotting times were prolonged in a dose-dependent manner by the addition of increasing amounts of vanadate to the clotting assay mixture. At a 2 mM concentration, vanadate prolonged the clotting time from 94 to 470 s (5 fold). The prolongation by heparin was 2.8 to 7 fold in the concentration range of 3—10 mU/ml (data not shown). The anticoagulant action of vanadate at 2 mM corresponded to that of heparin at 5 mU/ml. Amiloride is proposed to inhibit the action of vanadate on modulation of LPL activity in isolated rat fat pads through mechanisms of inhibitory action on tyrosine kinase activity of the insulin receptor.[6,7,15] The plasma, therefore, was incubated with vanadate or heparin in the presence of amiloride (Fig. 2). The anticoagulant action of vanadate linearly decreased with an increase in the concentration of amiloride and almost disappeared at 1 mM concentration of amiloride, but that of heparin was less preserved. Table I shows changes in the anticoagulant action of vanadate in the presence or absence of heparin. The actions of vanadate at 1 and 2 mM concentrations were

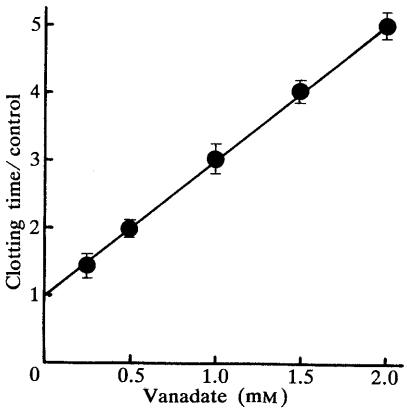

Fig. 1. Dose-Response Curve for Anticoagulant Action of Vanadate

Normal human plasma was preincubated at the indicated concentrations of vanadate in 50 mM Tris–HCl buffer, pH 7.9, containing 50 mM NaCl at 37 °C for 15 min, and kaolin-induced clotting times were determined as described under Materials and Methods.

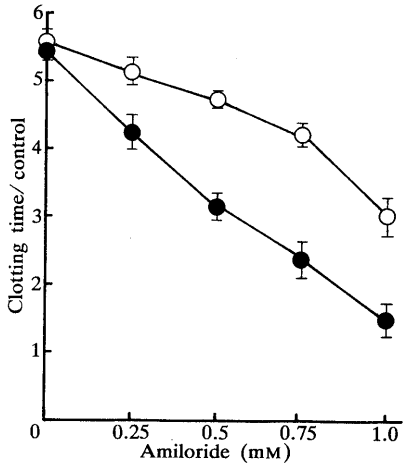

Fig. 2. Effect of Amiloride on Anticoagulant Actions of Vanadate and Heparin

The plasma was preincubated with vanadate (2 mM, ●) or heparin (5 mU/ml, ○) at the indicated concentrations of amiloride at 37 °C for 15 min.

Table I. Anticoagulant Action of Vanadate in the Presence or Absence of Heparin

	Clotting time/control	
	With heparin	Without heparin
Control	2.9±0.1	1.0
Vanadate (1 mM)	5.7±0.2	2.6±0.1
Vanadate (2 mM)	8.4±0.3	5.7±0.2

The plasma was preincubated with vanadate at 37°C for 15 min in the presence or absence of heparin (2.5 mU/ml).

completely additive to that of heparin at the concentration of 2.5 mU/ml. Next, several compounds, which inhibited the stimulatory effect of vanadate on LPL[16] and insulin-sensitive cyclic AMP phosphodiesterase activities in isolated rat fat pads to various extents (in preparation for publication), were tested. When plasma was preincubated with genistein (50 μM), biochanin A (100 μg/ml), dibutyryl cyclic AMP (3 mM), or 3-isobutyl-1-methylxanthine (1 mM), these compounds never showed any inhibition of the anticoagulant action of vanadate (data not shown).

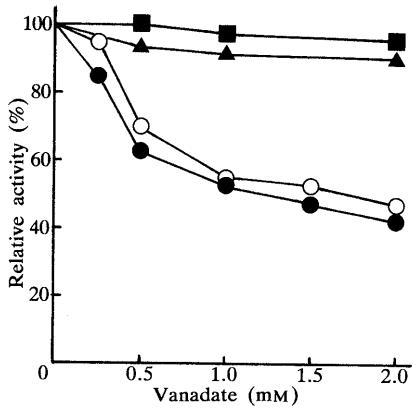

Fig. 3. Effect of Vanadate on Amidolytic Activities of Factor Xa and Thrombin

Factor Xa (0.4 mg/ml) was preincubated at the indicated concentrations of vanadate without amiloride (●) or with (1 mM, ■), and thrombin (0.2 μg/ml) at the indicated concentrations of vanadate without amiloride (○) or with (1 mM, ▲) at 37 °C for 15 min.

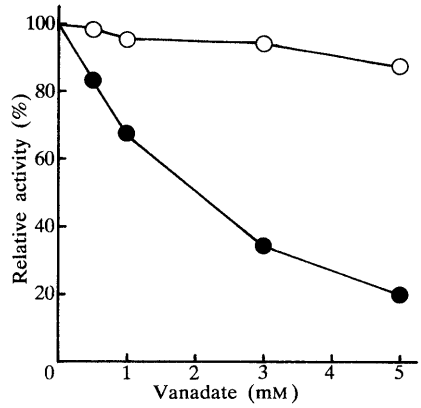

Fig. 4. Effect of Vanadate on Amidolytic Activities of Trypsin and α-Chymotrypsin

Trypsin (1 mg, ●) and α-chymotrypsin (1 mg, ○) were preincubated at the indicated concentrations of vanadate at 37 °C for 15 min, and their activities were determined as described under Materials and Methods.

Addition of vanadate to electropermeabilized human platelets caused an increase in tyrosine phosphorylation of proteins and stimulated the secretion of serotonin and platelet-derived growth factor.[17] When intact human platelets were incubated with a combination of vanadate and H_2O_2, tyrosine phosphorylation of proteins and aggregation was stimulated.[18] However, vanadate or H_2O_2 alone did not show these effects on intact human platelets. These results suggest that mechanisms other than its effect on platelets are involved in the anticoagulant action of vanadate. It is known that many proteolytic enzymes are involved in the coagulation cascade which activates coagulation factors.[19] Of these factors, factor Xa, converting prothrombin to thrombin, and thrombin, activating factor XIII, are key enzymes for the regulation of clotting. Therefore, the effect of vanadate on the amidolytic activity of these two enzymes was investigated. The amidolytic activity of each progressively decreased with an increase in the concentration of vanadate, yet neither decrease was observed in the presence of amiloride (Fig. 3).

Little or no decrease in either amidolytic activity was observed with heparin (data not shown). The human

placental anticoagulant protein, a new member of lipocortin, inhibits coagulation by binding specifically to phospholipid vesicles but not by binding to factor Xa or prothrombin.[10] Thus, vanadate seems to show prolongation of the clotting time of plasma through mechanisms differing in part from the actions of heparin and lipocortin. Factor Xa and thrombin are reported to be serine-protease, carrying the catalytically essential serine and histidine functionalities and showing trypsin-like specificities on small substrates.[19] Therefore, the effect of vanadate on the amidolytic activity of trypsin and α-chymotrypsin was compared. When trypsin or α- chymotrypsin was incubated with vanadate, the amidolytic activity of trypsin was decreased by the addition of increasing amounts of vanadate to the incubation mixture, but the activity of α-chymotrypsin was not decreased (Fig. 4). These results suggest that vanadate preferentially inhibits amidolytic activities of trypsin and trypsin-like enzymes. A preliminary report showed that vanadate (10 mM) strongly suppressed endogenous or exogenous protein degradation in isolated rat hepatocytes, and that the suppression would be due to a direct inhibition of the lysosomal cathepsins.[20] However, further detailed mechanisms of vanadate action as a proteinase inhibitor are still unknown. Amiloride is pyrazinoylguanidine bearing amino groups on the 3- and 5-positions and a chloro group on the 6-position of the pyrazine ring, and its guanidino moiety is protonated at physiological pH.[21] Arginine, carrying a guanidino group, is included both in synthesis and in polypeptide substrates for thrombin and trypsin.[22] It also is proposed that *p*-nitrophenyl-*p'*-guanidinobenzoate is the best compound available as a specific active site titrant for thrombin.[22] The protonated guanidino group in amiloride may play an important role in protecting the active site of the enzyme from attack by vanadate and antithrombin III activated by heparin. Therefore, there is the possibility that vanadate inhibits the activity by binding to the enzyme. However, further detailed mechanisms remain to be elucidated.

In conclusion, vanadate appears to show its anticoagulant action through mechanisms involving in part the inhibition of the activity of both factor Xa and thrombin, and differing from the action *via* the vanadate-sensitive pathway in isolated rat fat pads.

References

1) N. D. Chasteen, *Struct. Bonding*, **53**, 107 (1983).
2) B. R. Nechay, *Annu. Rev. Pharmacol. Toxicol.*, **24**, 501 (1984).
3) S. Tamura, T. A. Brown, J. H. Whipple, Y. Fujita-Yamaguchi, R. E. Dubler, K. Cheng, and J. Larner, *J. Biol. Chem.*, **259**, 6650 (1984).
4) J. Meyerovitch, Z. Farfel, J. Sack, and Y. Shechter, *J. Biol. Chem.*, **262**, 6658 (1987).
5) W. C. Duckworth, S. S. Solomon, J. Liepnieks, F. G. Hamel, S. Hand, and D. E. Peavy, *Endocrinology*, **122**, 2285 (1988).
6) H. Ueki, M. Sera, and K. Tanaka, *Arch. Biochem. Biophys.*, **272**, 18 (1989).
7) M. Cupp, A. Bensadoun, and K. Melford, *J. Biol. Chem.*, **262**, 6383 (1987).
8) E. W. Davie and K. Fujikawa, *Annu. Rev. Biochem.*, **44**, 799 (1980).
9) R. D. Rosenberg, "Heparin," ed. by I. Witt, Walter de Gruyter, Inc., Berlin, 1983, pp. 3—33.
10) T. Funakoshi, R. L. Heimark, L. E. Hendrickson, B. A. McMullen, and K. Fujikawa, *Biochemistry*, **26**, 5572 (1987).
11) K. Fujikawa, M. E. Legaz, and E. W. Davie, *Biochemistry*, **11**, 4892 (1972).
12) T. Morita, H. Kato, S. Iwanaga, K. Takada, T. Kimura, and S. Sakakibara, *J. Biochem.* (Tokyo), **82**, 1495 (1977).
13) M. Muramatsu, T. Itoh, M. Takei, and K. Endo, *Biol. Chem. Hoppe-Seyler*, **369**, 617 (1988).
14) H. Sawada, H. Yokosawa, M. Hoshi, and S. Ishii, *Experientia*, **39**, 377 (1983).
15) R. J. Davis and M. P. Czech, *J. Biol. Chem.*, **260**, 2543 (1985).
16) M. Sera, K. Tanaka, T. Morita, and H. Ueki, *Arch. Biochem. Biophys.*, **279**, 291 (1990).
17) K. M. Lerea, N. K. Tonks, E. G. Krebs, E. H. Fischer, and J. A. Glomset, *Biochemistry*, **28**, 9286 (1989).
18) T. Inazu, T. Taniguchi, S. Yanagi, and H. Yamamura, *Biochem. Biophys. Res. Commun.*, **170**, 259 (1990).
19) L. Lorand, "Methods Enzymology," Vol. 45, ed. by L. Lorand, Academic Press, Inc., New York, 1976, pp. 31—176.
20) P. O. Seglen and P. B. Gordon, *J. Biol. Chem.*, **256**, 7699 (1981).
21) T. R. Kleyman and E. J. Cragoe, Jr., *J. Membrane Biol.*, **105**, 1 (1988).
22) S. Magnusson, "The Enzymes," Vol. 3, ed. by P. D. Boyer, Academic Press, Inc., New York, 1971, pp. 277—321.