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Antithrombotic effects of synthetic peptides targeting various functional domains of thrombin

(arterial thrombosis/anion-binding exosite/catalytic site/baboon models)

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ABSTRACT To determine *in vivo* functional roles for thrombin's structural domains, we have compared the relative antithrombotic and antihemostatic effects of (*i*) catalytic-site antithrombin peptide, D-Phe-Pro-Arg; (*ii*) exosite antithrombin peptide, the C-terminal tyrosine-sulfated dodecapeptide of hirudin; and (*iii*) bifunctional antithrombin peptide, a 20-mer peptide combining catalytic-site antithrombin peptide and exosite antithrombin peptide with a polyglycyl linker. All three peptides inhibited thrombin-mediated platelet aggregation and fibrin formation *in vitro*. *In vivo* thrombus formation was measured in real time as ^{111}In-labeled platelet deposition and ^{125}I-labeled fibrin accumulation on thrombogenic segments incorporated into chronic exteriorized arteriovenous access shunts in baboons. Under low flow conditions, the continuous infusion of peptides reduced thrombus formation onto collagen-coated tubing by half at doses (ID_{50}) and corresponding concentrations (IC_{50}) of 800 nmol per kg per min and 400 nmol/ml for catalytic-site antithrombin peptide, >1250 nmol per kg per min and >1500 μmol/ml for exosite antithrombin peptide, and 50 nmol per kg per min and 25 nmol/ml for bifunctional antithrombin peptide. Under arterial flow conditions, systemically administered bifunctional antithrombin peptide decreased thrombus formation in a dose-dependent manner for segments of collagen-coated tubing or prosthetic vascular graft at ID_{50} and IC_{50} values of 120 nmol per kg per min and 15 nmol/ml; this dose also produced intermediate inhibition of hemostatic function [bleeding time, 21 ± 3 min vs. 4.5 ± 0.5 min (baseline values); $P < 0.001$; activated partial thromboplastin time, 285 ± 13 sec vs. 31 ± 3 sec (baseline), $P < 0.001$]. In contrast, thrombus formation onto segments of endarterectomized aorta was potently decreased by bifunctional antithrombin peptide with an ID_{50} value of 2.4 nmol per kg per min and an IC_{50} value of 0.75 nmol/ml, a systemic dose that failed to affect hemostasis. Thus, inhibiting both thrombin's catalytic and exosite domains increases antithrombotic potency by several orders of magnitude over the inhibition of either domain alone, particularly at sites of deep arterial injury.

Various structural domains, including the catalytic site and two flanking clusters of accessory binding sites, regulate thrombin's interactions with biological substrates (1–3). For example, thrombin activation of platelets requires binding via accessory domains to the receptor with subsequent catalytic receptor cleavage (4, 5). Similarly, the proteolytic conversion of fibrinogen to fibrin depends on the binding of thrombin to fibrinogen by a related exosite (6, 7). Conversely, the heparin-catalyzed inactivation of thrombin by plasma antithrombin requires simultaneous binding between a different accessory domain and the catalytic site of thrombin to form a termolecular complex (8–10). Thrombin also binds tightly with fibrin (11, 12) via accessory sites that appear to be shared by the antithrombin–heparin complex binding domains, thereby explaining resistance of fibrin-bound thrombin to inactivation by heparin (11–13). Importantly, fibrin-bound thrombin proteolytically activates platelets (14), cleaves fibrinogen (11), and is susceptible to inactivation by direct inhibitors of thrombin (14–16). To further probe thrombin's structure and function, domain-directed peptides have been synthesized and their properties have been compared *in vitro* and *in vivo*. The interactions of these peptides with thrombin have been established by x-ray crystallography (3, 18, 19). The baboon was used as the experimental thrombosis model because its hemostatic apparatus is similar to that of humans (14, 15, 20–22).

MATERIALS AND METHODS

Animals Studied. Twenty normal juvenile male baboons (*Papio cynocephalus*) weighing 8–16 kg were used in these studies. All procedures were approved by the Institutional Animal Care and Use Committee in compliance with the National Institutes of Health (23), Public Health Service policy, the Animal Welfare Act, and related university policies. Blood counts and hematocrits were measured on whole blood collected in EDTA (2 mg/ml), by using a J. T. Baker model 810 whole-blood analyzer. Template bleeding time measurements were performed on the shaved volar surface of the forearm as described (14, 15).

Surgically implanted exteriorized femoral arteriovenous (AV) access shunts were used for interposition of thrombogenic segments, drug infusions, and blood sampling (21). These AV shunts do not detectably activate platelets or fibrinogen (21). Thrombogenic segments were subsequently incorporated into the AV shunts of awake animals for 1 h, while the flow was measured through the shunt (C-clamp flow probe interfaced with a Transonic T206 blood-flow analyzer; Transonic, Ithaca, NY).

Models of Thrombosis. In low-flow studies, thrombus formation was measured onto segments of collagen-coated tubing (CT), 2 cm long and 4 mm i.d., incorporated into AV shunts with the blood flow controlled at 20 ml/min (shear rate, ≈100 sec^{-1}) by using a roller pump placed distal to the device as described (22, 24). Peptides were infused proximal to the device, to thereby attain sufficiently high local concentrations to produce inhibitory effects and to reduce the possibility of adverse systemic effects.

Abbreviations: CSAP, catalytic-site antithrombin peptide; ESAP, exosite antithrombin peptide; BAP, bifunctional antithrombin peptide; CT, collagen-coated tubing; VG, vascular graft; EA, endarterectomized aorta; AV, arteriovenous; APTT, activated partial thromboplastin time.

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High-flow studies (150–200 ml/min; shear rates, ≈750 sec^{-1}) were used to assess the relative antithrombotic and antihemostatic effects of continuous intravenously administered peptides for three thrombogenic segments:

(i) CT. Segments (2 cm long; 4 mm i.d.) of expanded poly(tetrafluroethylene) vascular graft (VG; internodal distance, 30 μm) were coated with bovine collagen type I as described (15, 22). The smooth luminal adherent layer of collagen fibrils was stabilized by cross-linking in 1% glutaraldehyde at 4°C for 12 h. Before use, the grafts were washed extensively with buffered saline and coupled to silicone rubber tubing to form a smooth configuration for incorporation into the AV shunt system (15).

(ii) Dacron VG. Dacron graft (i.d., 4 mm) crimped, knitted, and externally supported (Sauvage external velour; mean porosity, 2000–2200 ml of H_2O per min at 120 mmHg) was obtained from C. R. Bard (Billerica, MA). Segments 5 cm long were rendered impervious to blood leakage by an external wrapping of Parafilm (American Can Company, New York) and "heat shrink" Teflon tubing (5.3 mm i.d.). Butt joints were constructed that ensured smooth luminal flow surface (14, 15, 20).

(iii) Endarterectomized aorta (EA). Normal baboon aorta was removed in 4-cm segments by sharp dissection and surgically endarterectomized as described (15, 24). Flanged Teflon (Small Parts, Miami), 4 mm i.d., was used to cannulate each end of the aortic segment and secured by silk ligatures. Then, a shrinkable Teflon sleeve encased each endarterectomized aortic segment, constraining the vascular segment in a cylindrical configuration with a smooth transition from tubing to vessel suitable for incorporation into the AV shunts (15, 24).

Measurements of Thrombosis. Autologous platelets were labeled with [^{111}In]indium oxine (Amersham), as described (25), and infused into the animal at least 1 h before initiating studies. It has been shown that labeled platelets function normally (26). ^{111}In-labeled platelet deposition onto thrombogenic segments was measured by a GE 400T γ camera (General Electric) interfaced with an MDS computer system supported by A^3 image-processing software (Medasys, Ann Arbor, MI). Images were acquired at 5-min intervals and ^{111}In-labeled platelet deposition was determined by subtracting the whole-blood radioactivity contained in each segment from each dynamic image. The total number of platelets deposited (labeled and unlabeled) was calculated by dividing the deposited ^{111}In-labeled platelet activity by the blood standard platelet activity and multiplying by the volume of the blood standard and the circulating platelet count (platelets per ml). The results were expressed as platelets deposited per cm (14, 15). To determine fibrin accumulation, homologous ^{125}I-labeled fibrinogen (5 μCi; 1 Ci = 37 GBq) was prepared as described (15, 22) and injected at least 15 min before starting the studies. The ^{125}I-labeled fibrinogen was shown to be 95% clottable and functionally equivalent to unlabeled fibrinogen. Thrombogenic segments were obtained at the conclusion of each study, washed, and stored for 30 days until the ^{111}In activity had decayed. Then, the incorporated radioactivity was measured in a γ counter. Deposited fibrin (mg) was calculated by dividing the ^{125}I activity (cpm) in each segment by the coagulable plasma ^{125}I activity (cpm/ml) and multiplying by the plasma fibrinogen level (mg/ml) (16, 22). Results were expressed as total fibrin (mg/cm). The concentration of fibrinogen in plasma was estimated spectrophotometrically by a modification of Jacobsson's method (27). Blood tests of thrombosis were also determined by RIA as follows: (*i*) plasma levels of platelet-specific α-granule protein platelet factor 4 and of β-thromboglobulin and (*ii*) fibrinopeptide A, a thrombin cleavage product of fibrinogen (15, 20).

Domain-Directed Antithrombin Peptides. Catalytic site antithrombin peptide (CSAP; D-Phe-Pro-Arg), exosite antithrombin peptide (ESAP), also called "hirugen" (28, 29) [Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(OSO_3)-Leu, the sulfated C-terminal dodecapeptide of hirudin], and bifunctional anti-thrombin peptide (BAP), also called "Hirulog-1" (17, 30) [D-Phe-Pro-Arg-Pro-$(Gly)_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu], were prepared by solid-phase synthesis as described (30, 31). Peptides were purified by reverse-phase HPLC to >95% homogeneity and characterized by amino acid analysis, fast atom bombardment–mass spectrometry, and/or automated Edman degradation. When CSAP, ESAP, and BAP were studied in the low-flow thrombogenic device, they were infused 30 cm proximal to the collagen-coated segment to ensure adequate mixing with blood. In the high-flow configuration, the peptides were infused intravenously for 10 min before thrombogenic surfaces were interposed and then for another 60 min while measuring thrombus accumulation. Inhibition of thrombin's amidolytic activity was measured for CSAP, ESAP, and BAP by using a chromogenic substrate (Spectrozyme TH; American Diagnostica, Greenwich, CT), and kinetic measurements were performed as reported (17). The *in vitro* anticoagulant activities of CSAP, ESAP, and BAP were measured by thrombin time assays using pooled normal human plasma (George King Biomedical, Overland Park, KS). Assays employed a range of peptide concentrations and a fixed concentration of thrombin (0.5 μg/ml). Plasma antithrombin activity levels of each peptide were determined in plasma prepared from blood collected in 3.8% sodium citrate [9:1 (vol/vol)] and immediately frozen at −70°C until assayed. The plasma concentrations of peptide were assayed as antithrombin activity levels in relationship to standard curves for each peptide produced in autologous pretreatment plasma, as described (14, 15). The inhibitory effects of the peptides on thrombin-induced platelet aggregation were investigated using gel-filtered human platelets as reported (14, 30) with a final thrombin concentration of 0.08 μg/ml.

Statistical Analysis. Statistical analyses used Student's *t* test (two-tailed) for paired and unpaired sample groups when the data were normally distributed (32). Otherwise results were compared using the nonparametric Wilcoxon signed rank test (32). All data in the results section are given as the mean ± SEM.

RESULTS

***In Vitro* Effect of CSAP, ESAP, and BAP.** The effects of the three antithrombin peptides toward thrombin-catalyzed hydrolysis of a chromogenic substrate were measured over a range of substrate concentrations, and the results were kinetically analyzed (17). The K_i values for CSAP and BAP

Table 1. Antithrombin, anticoagulant, and antiplatelet properties of domain-specific peptides

	K_i, μM		Thrombin	TT_{200},	IC_{50} Plts,
Peptide	Th	Try	specificity	μM	μM
CSAP	61.0	2300	37.7	51.4	32.6
ESAP	>2000	ND	ND	1.0	27.9
BAP	0.001	1400	1×10^6	0.016	0.025

K_i values for thrombin-catalyzed hydrolysis (Th) of Spectrozyme TH were measured as reported (17) and for trypsin-catalyzed hydrolysis (Try) of Spectrozyme TH at ionic strength 0.15 (pH 8.4) were measured in a borate buffer at 22–23°C. Thrombin specificity is the ratio of the K_i values for thrombin-catalyzed hydrolysis to trypsin-catalyzed hydrolysis. TT_{200} is the concentration of peptide required to prolong thrombin (0.5 μg/ml) clotting time of normal human plasma to 200% of control, determined by dose–response measurements and least-squares fit analysis of the data. The IC_{50} Plts is the concentration of peptide required to inhibit thrombin (0.08 μg/ml)-induced aggregation of gel-filtered human platelets to 50% baseline values. ND, not determined. ESAP failed to inhibit thrombin amidolytic activity at >2000 μM.

were 61 μM and 2.3 nM, respectively (Table 1). Consistent with its binding to a noncatalytic accessory site, ESAP failed to inhibit thrombin's cleavage of the chromogenic substrate at concentrations $\leq$2 mM. The effects of the antithrombin peptides on thrombin time clotting assays were also compared. BAP was three and ESAP one order of magnitude more potent than CSAP. The antithrombin peptides also inhibited thrombin-induced platelet aggregation. Although directly inactive toward thrombin catalytic site functions, ESAP indirectly inhibited cleavage/activation of fibrinogen and platelets *in vitro* by blocking the prerequisite substrate binding at the exosite. BAP was three orders of magnitude more potent than either of the other two peptides (IC_{50} of 25 nM vs. 30 μM). Thus, combining reactivities to both binding domains markedly increased potency.

***In Vivo* Antithrombotic Effect of CSAP, ESAP, and BAP.** To compare the antithrombotic potencies of CSAP, ESAP, and BAP under low-flow conditions for collagen-induced platelet-dependent thrombus formation, dose–response effects were measured for ^{111}In-labeled platelets and ^{125}I-labeled fibrin deposition onto segments of CT and compared with control studies (Fig. 1). Three groups (one for each peptide) of four animals were studied at different times and in random order.

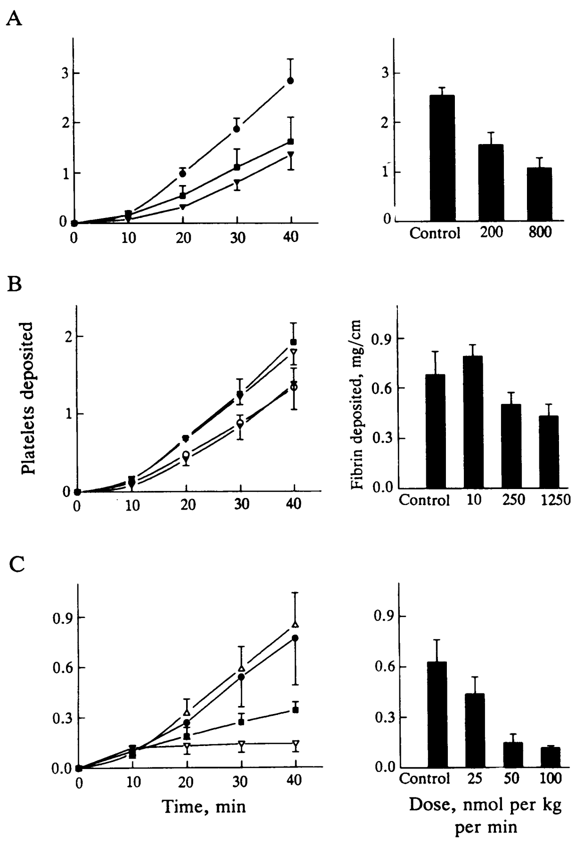

FIG. 1. Antithrombotic effect of synthetic antithrombin peptides targeting different domains of thrombin. Rate and extent of platelet deposition expressed as number ($\times 10^{-9}$) (*Left*) and accumulation of fibrin at 60 min (*Right*) for segments of CT incorporated as extension pieces in exteriorized chronic femoral AV access shunts placed in baboons and maintained under low-flow conditions with native unanticoagulated blood are shown. Dose–response curves for the three antithrombin peptides administered by continuous infusion into the shunt proximal to the collagen-coated segment are compared in *A* (for CSAP), *B* (for ESAP), and *C* (for BAP). Each value (mean ± SEM) is obtained from four animals. (*A*) ●, Control; ■, 200 nmol per kg per min; ▼, 800 nmol per kg per min. (*B*) ■, Control; ▽, 10 nmol per kg per min; ○, 250 nmol per kg per min; ▼, 1250 nmol per kg per min. (*C*) △, Control; ●, 25 nmol per kg per min; ■, 50 nmol per kg per min; ▽, 100 nmol per kg *per min*.

During control studies, ^{111}In-labeled platelets and corresponding amounts of ^{125}I-labeled fibrin accumulated rapidly onto CT during the period of exposure to flowing blood. Two doses of CSAP were infused for 40 min proximal to the CT segment and local intradevice concentrations of CSAP were estimated from the rate of drug infusion divided by the rate of flow in the shunt. Since the peptides were cleared within minutes, the intradevice concentrations were increased ≈10-fold over the systemic levels. Infusion of 800 nmol per kg per min prolonged activated partial thromboplastin time (APTT) values in blood effluent from the low-flow device (APTT, 104 ± 2 sec vs. 33 ± 2 sec; Table 2] and produced intermediate inhibition of thrombus formation on CT at plasma concentration of 400 nmol/ml (Fig. 1). An infusion of 200 nmol per kg per min had no detectable effects (Table 2 and Fig. 1). Despite the high local concentrations of ESAP in the low-flow system (Table 2), no significant inhibition of platelet deposition was observed, although fibrin accumulation was decreased (Fig. 1). In contrast, BAP potently interrupted platelet and fibrin deposition onto CT. Intermediate effects on thrombus accumulation were produced by BAP at 25 nmol/ml (50 nmol per kg per min), and complete inhibition occurred with BAP at 50 nmol/ml (100 nmol per kg per min). The local infusion of peptide produced systemic BAP levels of 20–25% of the local concentration (Table 2). From these dose–response data, the systemic ID_{50} antithrombotic dose of BAP for CT was estimated to be 100 nmol per kg per min.

***In Vivo* Effect of Systemically Administered Peptides.** Under arterial flow conditions, thrombogenic segments accumulated occlusive amounts of platelets and fibrin by 60 min of blood exposure (Fig. 2). Maximal values at 60 min were 3.3 ± 0.69 $\times 10^9$ platelets per cm for CT, 2.29 ± 0.49 $\times 10^9$ platelets per cm for VG, and 1.33 ± 0.33 $\times 10^9$ platelets per cm for EA. Thrombus formation was accompanied by transient declines in platelet counts (390 ± 30 to 340 ± 45 $\times 10^3$ platelets per μl) and by greatly increased plasma levels of blood markers of thrombus formation *in vivo* (Table 3). When infused systemically at doses of 400 nmol per kg per min, infusions of CSAP and ESAP had no detectable antithrombotic effects on platelet-dependent processes, presumably related to the low circulating concentration (i.e., ≈10% the level produced by infusing the same dose locally into the device). For example, during systemic infusions of CSAP, thrombus accumulated onto VG at a rate equal to controls (6.1×10^{-9} vs. 5.4×10^{-9} platelets per cm). Measurements of bleeding times and APTTs were also unaffected relative to control values (31 ± 1 vs. 33 ± 1 sec and 4.5 ± 0.5 vs. 5.0 ± 0.5 min, respectively).

BAP was infused systemically at 100 and 200 nmol per kg per min while segments of CT and VG were interposed into the AV shunt (Fig. 2). The blood levels of BAP remained constant throughout the period of study (Table 4), and no

Table 2. Effect of antithrombin peptides on coagulation

Peptide	Infusion, nmol per kg per min	Plasma concentration, nmol/ml	APTT, sec
CSAP	0	0	32 ± 2
	200	100	72 ± 6
	800	400	104 ± 2
	1600	800	124 ± 4
ESAP	0	0	33 ± 4
	9	5	44 ± 2
	250	175	86 ± 9
	1250	875	129 ± 13
BAP	0	0	33 ± 1
	25	13	280 ± 25
	50	25	>300
	100	50	>300

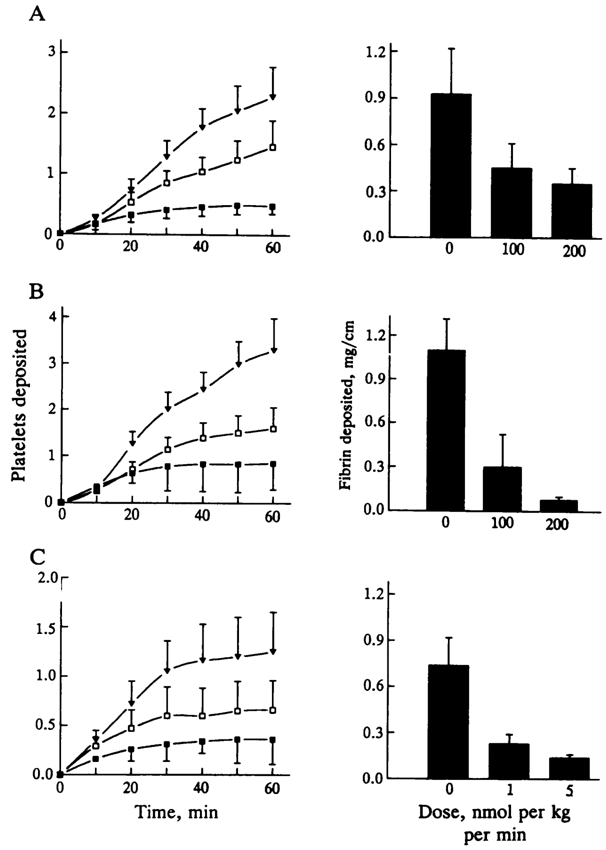

FIG. 2. Antithrombotic effect of BAP for CT, VG, and EA under arterial flow conditions. The dose–response effects of intravenously infused BAP are shown for platelet deposition expressed as number ($\times 10^{-9}$)(*Left*) and fibrin accumulation (*Right*) with native unanticoagulated blood flowing under arterial conditions for segments of CT (*A*), VG (*B*), and EA (*C*). Maximal antithrombotic effects are associated with marked effects on hemostatic function for CT and VG. Platelet deposition onto segments of EA is abolished by BAP infused intravenously at substantially lesser rates than those required for collagen or VG (i.e., 5 nmol per kg per min), doses that fail to affect platelet hemostatic function as measured by bleeding time measurements. Results are from five animals (mean ± SEM). (*A* and *B*) ▼, Control; □, 100 nmol per kg per min; ■, 200 nmol per kg per min. (*C*) ▼, Control; □, 1 nmol per kg per min; ■, 5 nmol per kg per min.

detectable effects on heart rate or blood pressure were observed. The higher dose of 200 nmol per kg per min gave a plasma level of 34 ± 4.9 nmol/ml and increased bleeding time to >30 min and APTT to >300 sec. The intermediate dose of 100 nmol per kg per min produced intermediate changes in hemostatic function. Systemic BAP potently inhibited the formation of thrombus induced by segments of EA; i.e., a dose of 5 nmol per kg per min markedly reduced platelet deposition with intermediate effects by 1 nmol per kg per min (Fig. 2).

Table 3. Effects of BAP on blood markers of thrombosis

Test	Plasma level baseline	Plasma levels after 60-min infusion		
		0	100	200
βTG, μg/liter	9.0 ± 3.8	37.0 ± 6.4	7.8 ± 2.3	10.3 ± 2.4
PF4, μg/liter	9.3 ± 2.9	31.0 ± 7.9	8.6 ± 1.3	7.2 ± 2.2
FPA, pmol/liter	5.5 ± 0.9	26.0 ± 4.8	4.5 ± 0.6	6.1 ± 1.6

These changes were produced by VG segments in exteriorized femoral AV shunts. BAP at 0, 100, or 200 nmol per kg per min was infused for 60 min and the plasma levels of the platelet-specific α-granule proteins β-thromboglobulin (βTG), platelet factor 4 (PF4), and fibrinopeptide A (FPA) were determined.

DISCUSSION

This study demonstrates that synthetic BAP, directed against both the catalytic and exosite domains of thrombin, produces potent dose-dependent antithrombotic effects of platelet-rich arterial-type thrombosis in primate models. In contrast, neither of the component peptide sequences, CSAP or ESAP, directed against the catalytic and exosite domains, respectively, exhibits useful antithrombotic effects *in vivo*. Platelet and coagulant hemostatic functions are impaired by BAP at doses that are antithrombotic for segments of CT and VG. However, BAP interrupts thrombus formation at sites of endarterectomy with greatly increased potency, thereby producing antithrombotic effects using systemic doses of BAP that do not compromise hemostasis.

Thrombin is the central enzyme mediating the formation of vascular thrombi and hemostatic plugs in response to vessel and tissue damage (1, 14–16). Moreover, fibrin-bound thrombin largely mediates platelet recruitment, giving rise to thromboocclusive complications occurring at sites of atherosclerotic stenosis undergoing spontaneous fissuring (33, 34) or interventional mechanical vascular procedures (35–37). Thrombin-mediated catalysis of fibrinogen is dependent on the anion-binding exosite, as shown by the fact that occupancy of this domain by the dodecapeptide derived from residues 53 to 64 of hirudin (ESAP) inhibits fibrinogen binding and subsequent fibrin generation *in vitro* (11, 28), with an IC_{50} value of ≈10 nmol/ml. Concordantly, *in vivo* ESAP reduces fibrin-dependent thrombus formation by 50% at a plasma concentration (IC_{50}) of ≈10 nmol/ml in low-flow venous-type thrombosis (Fig. 1). However, despite ESAP's inhibition of thrombin binding to its platelet receptor (4) and thrombin-induced platelet aggregation *in vitro* (29), with an IC_{50} value of ≈10 nmol/ml, ESAP fails to interrupt thrombin-mediated platelet-dependent thrombus formation in baboon models of high-flow arterial thrombosis at circulating concentrations >1 μmol/ml (Table 2). Moreover, despite high circulating levels of ESAP in these experimental animal studies, platelet hemostatic function is not impaired (Table 2), although blood clotting times are markedly prolonged (Table 2). Thus, the failure of ESAP to inhibit these thrombin-dependent processes indicates that they are independent of the anion-binding exosite *in vivo* or that such processes escape exosite blockade under physiologic conditions (38–40).

In this study the catalytic domain of thrombin is required for platelet activation and fibrinogen conversion to fibrin both *in vitro* and *in vivo* (Fig. 1), concordant with the potent effects of covalent inactivation of thrombin's catalytic site by D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (14). The peptides used in the present study were prepared and studied to probe more specifically the domain structure–function relationships of thrombin. The tripeptide D-Phe-Pro-Arg (CSAP) inhibits thrombin's amidolytic activity *in vitro* in a reversible manner with a K_i value of 61 μM. CSAP inhibits thrombin's induction of platelet aggregation and cleavage of fibrinogen *in vitro* with IC_{50} values of ≈30 μmol/ml and 100 μmol/ml, respectively. *In vivo*, the plasma levels of 100 nmol/ml produced half-maximal reduction in thrombus formation (Fig. 1). BAP was synthesized to combine the structural specificity of ESAP with the active-center specificity of CSAP. The resultant bifunctional peptide exhibits striking cooperation regarding the specificity and the potency for inhibition of both fibrin generation and platelet activation by thrombin *in vitro*. Moreover, BAP effectively interrupts both platelet-dependent and fibrin-dependent thrombus formation *in vivo* with at least a 10-fold greater potency than CSAP for VG and collagen segments (Fig. 1) and a 1000-fold greater potency for endarterectomy thrombosis (Fig. 2).

It is relevant to compare BAP's antithrombotic efficacy with respect to different thrombogenic segments incorpo-

Table 4. Effects of systemic BAP on hemostasis

Test	BAP dose administered						
	0	1	5	25	50	100	200
Plasma level, nmol/ml	—	0.5 ± 0.2	0.9 ± 0.3	2.5 ± 0.27	6.5 ± 0.7	12.0 ± 1.3	34.0 ± 4.9
Platelet count, no. ($\times 10^{-3}$)/μl							
Before	334 ± 29	409 ± 79	373 ± 23	300 ± 39	283 ± 19	302 ± 32	273 ± 68
After	292 ± 18	395 ± 52	347 ± 16	306 ± 37	260 ± 14	296 ± 25	234 ± 67
Bleeding time, min	4.3 ± 0.5	4.5 ± 1.0	5.6 ± 1.0	6.8 ± 2.0	14.5 ± 2.5	21.0 ± 2.0	>30
Fibrinogen, mg/ml							
Before	313 ± 23	322	274 ± 54	312 ± 20	267 ± 21	310 ± 15	361 ± 31
After	306 ± 17	318	277 ± 57	253 ± 13	237 ± 25	279 ± 15	322 ± 39
APTT, sec	33 ± 1	57 ± 6	97 ± 3	184 ± 22	252 ± 23	286 ± 8	>300

BAP doses administered, 0–200 nmol per kg per min, are shown.

rated into the baboon exteriorized AV shunts. The relative IC_{50} value for the EA thrombosis is two orders of magnitude lower than for the VG segments (Table 3), concentrations attained by administering intravenous infusions of 1–2 mg per kg per h into primates and readily achieved in humans (41). Presumably, the different concentrations of BAP required to produce half-maximal decreases in thrombus formation for each of these surfaces reflects the relative amounts of thrombin produced by the different thrombogenic devices. This interpretation suggests that mechanically damaged arteries exhibit thromboregulatory factors. The sensitivity of EA to BAP may be relevant to the concentrations needed to interrupt thrombus formation after mechanical vascular injury in humans, although this extrapolation may not be warranted for disrupted diseased vessels exhibiting highly thrombogenic elements, including tissue factor and collagen-rich extracellular matrix in atherosclerotic lesions (34).

It is important to compare relative antihemostatic risks and antithrombotic benefits for these domain-directed peptides. To establish meaningful relationships, the changes in hemostatic function (assessed by bleeding time and APTT measurements) are assessed at equipotent antithrombotic concentrations of peptide [i.e., the concentrations producing half-maximal decreases in thrombus formation (IC_{50})]. The observation that intermediate antihemostatic effects are observed for segments of CT and VG at concentrations that produce intermediate antithrombotic effects is noteworthy and indicates correspondence between the inhibition of thrombosis and hemostasis. Interestingly, complete interruption of EA thrombus formation is achieved at doses that do not affect hemostatic function.

Using domain-directed antithrombin peptides, we conclude that (*i*) inhibition of thrombin's catalytic site is essential to decrease platelet-dependent thrombosis *in vivo*, (*ii*) enhanced specificity is achieved through exosite binding, and (*iii*) combining catalytic-site inhibition with exosite specificity greatly amplifies antithrombotic potency.

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