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Effects of low molecular weight fibrin degradation products 6A and 6D on rabbit aorta strips

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Abstract

Two small molecular weight fibrin degradation product, the pentapeptide 6A and the undecapeptide 6D, produced relaxations of norepinephrine-contracted rabbit aorta strips. The relaxations were slow-developing and were elicited by both peptides at supramicromolar concentrations; the amplitude of relaxations were small for 6D. The relaxations induced by 6A were not dependent on the presence of endothelium and were not modified by a mixture of indomethacin, pyrilamine, and cimetidine. The amplitude of the relaxations produced by 6A and 6D increased as a function of incubation time *in vitro*.

In another experimental system, peptides 6A and 6D failed to increase 6-keto-$PGF_{1\alpha}$ release from cultured human umbilical endothelial cells. Histamine and bradykinin were both active in this system.

Introduction

It has been proposed that fibrin degradation products (FDP) of various molecular weight possess pharmacological functions. Gerdin and Saldeen [1] showed that dialysable, low molecular weight FDPs could increase vascular permeability following intradermal injection in rats; purification of the vasoactive material showed that a pentapeptide termed 6A (H-Ala-Arg-Pro-Ala-Lys-OH) and an undecapeptide called 6D (H-Ser-Gln-Leu-Gln-Lys-Val-Pro-Pro-Glu-Trp-Lys-OH) were the active compounds [2]. In various experimental systems, these compounds were shown to be vasoactive: they could increase vascular permeability [3–5], and in the case of 6A, relax vascular smooth muscle *in vitro* [6] and dilate coronary arteries *in vivo* [7]. The latter actions of 6A were partially or totally mediated by vasodilator prostanoids. Peptides 6A and 6D are presumably inactivated by kininase I (serum carboxypeptidase N) which splits the Phe-Arg bond in the bradykinin sequence; in addition peptide 6A is recognized by kininase II (angiotensin converting enzyme) [8] which splits the Pro-Phe bond of bradykinin.

It appeared useful to characterize further the actions of peptides 6A and 6D on the circulation by studying their effect on two vascular functions: the vasomotor response of the isolated rabbit vascular strips and prostacyclin (PGI_2) release from cultured human endothelial cells. These systems have been useful to analyze vascular actions of other peptides generated from blood, the kinins [9] and the anaphylatoxins [10, 11].

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Material and methods

Synthesis of peptides 6A, des Lys^5-6A, and 6D

Peptides were assembled by the solid phase method [12] on a 1% cross-linked polystyrene phenylacetamidomethyl (PAM) support [13] prepared in our laboratory. Esterification of the protected C-terminal to the PAM support was performed by means of the cesium salt procedure [14] and the substitution was evaluated by titration using the picric acid procedure, and by quantitative amino acid analysis. Substitution were found to be 0.42 mmol/g and 0.37 mmol/g for Boc-Ala-reasin and for Boc-Lys(2-chlorocarbobenzoxy)-resin, respectively. Syntheses were carried out on a 1 mmol scale using an automatic Kaeser Associates peptide synthesizer operated in the automatic mode. The cycles of deprotection-neutralization-coupling-washing were similar as described previously [15]. Boc (t-butyloxycarbonyl) derivatives were used for the temporary protection of the alpha-amino groups of amino acids. Benzyl (Bzl) ether, p-toluene sulfonyl (tosyl or Tos), 2-chlorocarbobenzoxy (2-Cl-Z) and cyclohexyl ester (Chx) were used for the protection of Ser, Arg, Lys and Glu reactive side chains, respectively. Preformed symmetrical anhydrides of the Boc derivatives (twofold excess of the anhydride) were used for coupling [15], except for Gln which was introduced by the dicyclohexylcarbodiimide/hydroxybenzotriazole procedure [16]. Coupling reactions were monitored by the ninhydrin test [17] and repeated if incomplete after 1 h. Boc protection was removed at each cycle using 33% trifluoroacetic acid (TFA) in dichloromethane and neutralization was performed by means of 5 repeated washes with 5% triethylamine in dichloromethane. After completion of the peptide sequence, the observed gain in weight corresponded in each case to a quantitative yield. Peptides were simultaneously cleaved from the resin support and deprotected using liquid HF in the presence of anisole (9:1 v/v) at 0° for 1 h. After evaporating the HF and washing the resin with ethyl acetate and ether, crude peptides were extracted with 25% acetic acid. The volume of the extract was then reduced under mechanical vacuum using a rotary evaporator to a ca. 15 ml volume and the concentrate immediately applied onto a G-15 Sephadex column (2.5 × 100 cm) equilibrated with 10% acetic acid: 490 mg, 399 mg, and 979 mg of desalted 6A, des-Lys^5-6A and 6D containing respectively 89%, 93% and 83% peptide were obtained, as determined by analytical reverse phase (Vydac octadecylsilane column, 5 micron, 4.6 × 250 mm) high performance liquid chromatography (hplc) (Waters) in a 0.1% TFA (A)-acetonitrile (B) system. Following a second and final step of purification using a preparative (500 mg scale) hplc system (Waters) operated under comparable conditions as determined by analytical hplc (peptide 6A: $T_r = 10$ min, 0–15% B in 20 min, flow rate 1.5 ml/min; peptide des-Lys^5-6A: $T_r = 12$ min, 0–30% B in 15 min, flow rate 1.5 ml/min; peptide 6D: $T_r = 14.6$ min, 10–40% B in 15 min, flow rate 1.5 ml/min), peptides 6A, des-Lys^5-6A and 6D were obtained as TFA salts with a purity of 99.5% minimum (reverse phase hplc), in yields of 76, 85 and 53%, respectively, based on the original Boc amino acid substitution on the resin. Quantitative amino acid analyses following acid (HCl 6N, 110° 20 h) and enzymatic (leucine aminopeptidase/pronase) hydrolysis corresponded to the expected amino acid ratio.

Isolated blood vessels

Segments of thoracic aorta were removed from New Zealand white rabbits (1.5–3 kg) killed by stunning and exsanguination. Fat and excess connective tissue were excised and helical strips were cut [18]. Tissues were tied at both ends and suspended in 5 ml organ baths containing oxygenated (95% O_2 and 5% CO_2) and warmed (37 °C) Krebs solution [19]. The strips were 15–20 mm long and 3–4 mm wide and submitted to a baseline tension of 2 g. Changes in tension resulting from applications of pharmacologically active substances were recorded by isometric transducers (model 52-9545, Harvard Bioscience, South Natick, MA) on a Harvard chart recorder (50-9315).

Protocols

Responses to peptides 6A and 6D were found to be vascular relaxations; norepinephrine-contracted tissues were used to record this effect in most experiments, as in [10]. After a 45 min equilibration period, norepinephrine (NE) was applied to a final concentration of 200 nM (ED_{30}–ED_{40}).

Fifteen min were allowed to develop the full contraction and reach a stable plateau; at this point test substances were applied in three or four increasing levels of concentration with sufficient time between each application for full development of the effect (cumulative concentration-effect curve). The same procedure was repeated at 3 hr and 6 hr (starting 15 min earlier with NE application) to assess the effect of long *in vitro* incubation on biological responses; rabbit vascular strips have been shown to increase considerably their responses to kinins under these conditions [9].

In experiments with inhibitory drugs, namely a mixture of pyrilamine, cimetidine and indomethacin, the tissues were exposed to a constant concentration of the drugs throughout the experiment. In other experiments, the endothelial cell lining of rabbit aorta strips was removed by gently scrubing the intimal surface with filter paper; the loss of acetylcholine (Ach)-induced relaxations in those preparations was monitored as an indication of a succesful procedure since endothelial cells are necessary for this response [20]. Paired controls from the same animal were obtained systematically in experiments dealing with inhibitory drugs or intimal damage.

Prostacyclin release by cultured endothelial cells

The experiments were performed on confluent 35 mm petri dishes of cultured endothelial cells derived from human umbilical veins as described [11]. The cells were not passaged and were used as primary cultures; at least three individual donors are represented in each experimental group. Cell counts obtained following a trypsin-EDTA treatment averaged 398000 per 35 mm dish.

The measurement of the prostacyclin decay product, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), was based on a commercial radioimmunological assay involving a tritiated tracer (New England Nuclear, Boston MA). After rinsing the dishes in a protein-free buffer (Earle's balanced salt solution), the output of 6-keto-$PGF_{1\alpha}$ was evaluated in each dish for two consecutive 15 min period by sampling the supernatant three times at 15 min intervals, as described [11]. Basal release was evaluated during the first period, and the second one represented the stimulated output since FDPs and other substances were applied immediately after the second sampling.

Drugs

(–)-Norepinephrine, pyrilamine maleate, histamine dihydrochloride, indomethacin, acetylcholine chloride, and bradykinin were purchased from Sigma (St. Louis, MO). Cimetidine HCl (Smith, Kline & French, Canada) was also used. Indomethacin was dissolved in 0.1 *M* Na_2CO_3. Other drugs were dissolved in pyrogen-free 0.9% saline or Earle's balanced salt solution and injected into the organ baths or petri dishes under a volume of 5–75 µl. Concentrations are expressed at the tissue or cell level and refer to the free substance.

Statistics

Results are expressed as means ± SEM. Student's paired t-test was used to compare the effect of treatments with paired controls.

Results

Effects of peptides 6A and 6D on rabbit aorta strips

The peptides 6A and 6D had no effect on the resting tone of rabbit aorta strips (Fig. 1). On preparations contracted with norepinephrine (NE, 200 n*M*), both peptides were able to induce relaxations when applied at relatively high concentrations (tracings, Fig. 1; concentration-effect rela-

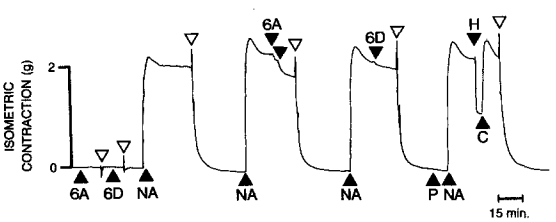

Figure 1
Effect of fibrin degradation products 6A and 6D on the resting and contracted rabbit isolated aorta. The preparation was contracted with noradrenaline (NA, 200 n*M*). Other substances applied were 6A, 6D (final concentration 52 µ*M* for both peptides), pyrilamine (P, 5 µ*M*), histamine (H, 61 µ*M*) and cimetidine (C, 12 µ*M*). Abscissa scale: time, ordinate scale, isometric contraction, g. Closed symbols refer to the application of stimulants and open symbols to washouts of the tissue.

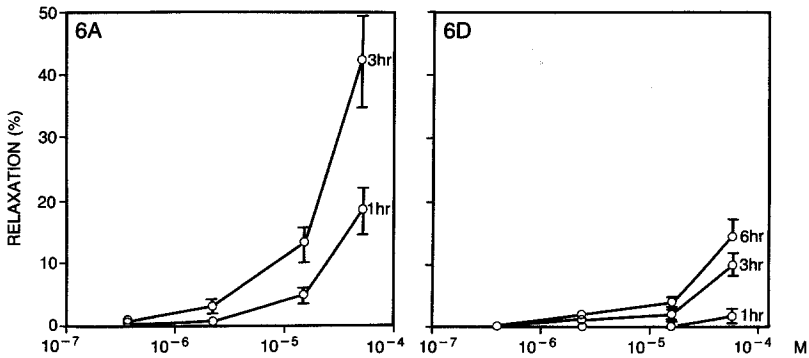

Figure 2
Concentration-effect relationship for 6A- and 6D-induced relaxations of rabbit aorta strips contracted with NE (200 n*M*). Results are expressed as mean ± S.E.M. of 12 or 23 determinations respectively for 6A and 6D.

Table 1
Effect of histamine applied to a plateau of contraction produced by norepinephrine (200 n*M*).

Incubation time	Concentration of histamine	Responses to histamine	
		Without pyrilamine ($n=5$–8)	With pyrilamine (5 μ*M*) ($n=4$)
1 hr	1 μ*M*	+14.7± 2.1[a]	− 4.0±0.8
	12 μ*M*	+47.7± 3.1	−22.2±3.1
	63 μ*M*	+66.0± 6.0	−49.3±2.7
3 hr	1 μ*M*	+10.5± 2.2	− 5.7±0.7
	12 μ*M*	+46.9± 5.5	−24.8±2.2
	63 μ*M*	+70.1± 7.2	−41.4±4.4
6 hr	1 μ*M*	+10.9± 4.6	− 5.1±0.8
	12 μ*M*	+42.7±14.4	−24.9±2.8
	63 μ*M*	+67.3±27.3	−38.3±6.5

[a] Results expressed as per cent of NE-plateau of contraction, either relaxation (negative value) or further contraction (positive value).

tionship, Fig. 2). The threshold for FDP-induced relaxations was over 1 μ*M* for both peptides, and important relaxations were obtained at 52 μ*M* for 6A; 6D-induced relaxations were highly variable from animal to animal and average out only weak effects. The development of FDP-induced relaxations was slower than histamine-induced relaxations recorded in the presence of pyrilamine (Fig. 1) or than acetylcholine-induced relaxations (not shown). The cumulative concentration-effect curves recorded at 1 and 3 hours from the beginning of *in vitro* incubation show an increase in the magnitude of the responses for both peptides (Fig. 2). It appears that the sensitivity of the preparations, expressed as the threshold concentration for relaxation, is not changed during the incubation; the magnitude of the response is amplified.

Peptides 6A and 6D have been described as histamine releasers [21] and their effects have been compared with this vasoamine. Histamine, applied over a NE-induced plateau, contracted further rabbit aorta strips; this contractile response, expressed as a percent of the NA plateau, remained fairly constant throughout the 6 hr incubation period (Table 1). The same concentrations of histamine applied on tissues continuously exposed to pyrilamine (5.0 μ*M*) promoted rapidly developing relaxations that could be reversed by the application of cimetidine (Fig. 1). These relaxant effects were also relatively constant over time when expressed as a percent of the NE-induced plateau (Table 1).

Studies on the mechanism of 6A-induced vascular relaxations were performed (Tables 2 and 3); 6D was not included in these studies due to its weak and inconsistant effects. The relaxing effect of peptide 6A on rabbit aorta strips was not dependent on the presence of the endothelial lining (Table 2). Strips with a rubbed intimal surface responded as much to 6A as paired controls. In the same series of experiments, intimal damage virtually abolished relaxations induced by acetylcholine (100 n*M*) applied on a NE-induced plateau of contraction.

Table 2
Effect of intimal damage on responses of rabbit aorta strips to peptide 6A.

Incubation time	Concentration	Responses to 6A ($n=6$)	
		Damaged tissues	Paired controls
3 hr	15 μM	− 6.5±1.9[a]	− 8.2± 2.0
	52 μM	−24.1±5.0	−26.9± 3.4
6 hr	15 μM	− 7.7±2.2	− 9.3± 3.2
	52 μM	−23.1±4.2	−29.3± 7.1
6.5 hr	Ach, 0.1 μM	+ 0.8±0.8[b,*]	−46.1±10.5

[a] Relaxation in % of NE-induced plateau (200 nM). Responses obtained in tissues submitted to intimal damage were compared with those elicited on paired controls. * $p<0.01$.
[b] Ach (100 nM) was applied on a NE-induced plateau. The relaxation observed is expressed as % of plateau.

Table 3.
Effect of a mixture of antagonists on responses of rabbit aorta strips to peptide 6A.

Incubation time	Concentration	Responses to 6A ($n=7$)	
		With antagonists	Paired controls
3 hr	15 μM	− 4.3±1.0[a]	− 5.4±0.9
	52 μM	−17.9±2.7	−19.5±3.1
6 hr	15 μM	− 5.7±1.4	− 6.0±1.4
	52 μM	−17.1±4.6	−18.5±4.4

[a] Relaxation in % of NE-induced plateau (200 nM). Responses obtained in tissues submitted to intimal damage were compared with those elicited on paired controls. No significant differences were found. Treated tissues were exposed to a mixture of pyrilamine (5 μM), cimetidine (12 μM) and indomethacin (5.6 μM).

Continuous exposure of tissues to a mixture of pyrilamine (5 μM), cimetidine (12 μM) and indomethacin (5.6 μM) did not modify relaxations induced by peptide 6A (Table 3). The concentrations of pyrilamine and cimetidine used were sufficient to abolish all effects of histamine on the rabbit aorta preparation.
The fragment des-Lys[5]-6A was completely inactive as a relaxant of the rabbit aorta up to 240 μM. Peptides 6A and 6D could also relax histamine-induced contractions of the rabbit aorta (not shown) suggesting that they are not alpha-adrenergic blockers.

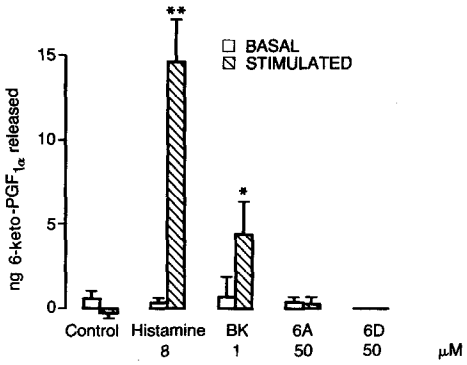

Figure 3
Release of 6-keto-$PGF_{1\alpha}$ by dishes of endothelial cells during two consecutive 15 min periods. Test substances or their vehicle (75 µl Earle's solution) were applied to cultures at the beginning of the second (stimulated) period. Results are means of 5–6 determinations. Differences between basal and stimulated rates of release are indicated by $*p<0.05$ and $**p<0.001$.

6-keto-$PGF_{1\alpha}$ release by cultured endothelial cells

Both low molecular weight fibrin degradation products 6A and 6D at 50 μM failed to promote 6-keto-$PGF_{1\alpha}$ release over baseline level in dishes of human endothelial cells (Fig. 3). Histamine (8 μM) was included as a positive control and was highly effective to promote prostacyclin release, as reported previously [11]. Bradykinin (1 μM) could also increase significantly 6-keto-$PGF_{1\alpha}$ output from human umbilical endothelial cells, as shown by several investigators [22, 23].

Discussion

Two small peptides formed by plasmin during fibrinolysis and termed 6A and 6D by Belew et al. [2] were found to be vascular smooth muscle relaxants in the present experiments. Rabbit aorta relaxations induced by 6A show distinctive features. Firstly, they are endothelium-independant unlike those elicited on the same preparation by acetylcholine, adenine nucleotides and the peptide substance P [20]; therefore the postulated "endothelium-dependent relaxing factor" does not appear to be involved in the vasodilator action of 6A. Secondly, the relaxations induced by FDP occur at relatively high concentrations. All reported actions of 6A and 6D, such as the permeability effect in the human skin [5] and the

6A-induced relaxation of the isolated bovine mesenteric artery [6], occur at comparably high concentrations: over 1 μM. The present experiments do not identify a more sensitive bioassay. This could be taken as evidence against any physiologic role for these basic peptides if it cannot be proved that such high levels are reached *in vivo*. Alternatively, the short sequences identified as 6A and 6D may not be the optimal stimulants for their putative vascular receptors. At this point, it is not clear whether the two peptides possess receptors in tissues, or even share a common receptor.

Another aspect of FDP action on rabbit aorta strips is remarkable: a considerable development of the relaxant responses to 6A and 6D from a small initial response was shown. This process was also observed on isolated smooth muscle preparations selectively sensitive to des-Arg metabolites of kinins (possessing the B_1-type of receptors for kinins) [9]. In the present experiments, 6A and 6D do not stimulate kinin receptors, since bradykinin and des-Arg^9-bradykinin contract this preparation, even if it has been pre-contracted with NE (not shown). The mechanism of the response increase as a function of incubation time remains to be established.

Finally, it should be stressed that rabbit aorta responses to 6A and 6D are independant of local histamine release and of cyclo-oxygenase products. This contrasts with the permeability effect of these peptides in rats which was inhibited by an antihistamine [1]; both peptides, as other basic compounds, could also release histamine from rat mast cells [21]. 6A-induced relaxation of bovine mesenteric arteries was abolished by indomethacin pretreatment [6]; 6A-induced coronarodilation *in vivo* in the dog was also partially inhibited by indomethacin [7]. Vasodilator prostanoids such as prostaglandins E_2 and I_2 are not active on the isolated rabbit aorta [24] and this may account for the resistance of FDP effects to indomethacin in the present experiments. On the other hand peptides 6A and 6D failed to promote the release of prostacyclin, the most important cyclooxygenase metabolite produced by human umbilical endothelial cells. This suggests that FDP may not promote directly the release of vasodilator prostanoids from the endothelium.

In conclusion, the basic peptides 6A and 6D may alter some vascular functions by various mechanisms, some of which are uncharacterized, but do so only at high concentrations. Other blood-derived peptides, the kinins and the anaphylatoxins, affect vascular strips at much lower concentrations [9, 10, 19].

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