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NOVEL PEPTIDES DERIVED FROM A REGION OF LOCAL HOMOLOGY BETWEEN UTEROGLOBIN AND LIPOCORTIN-1 INHIBIT PLATELET AGGREGATION AND SECRETION

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The active site for uteroglobin inhibition of phospholipase A_2 has been localized to a nonapeptide (P1) which is partially homologous to a nonapeptide (P2) in lipocortin, which also inhibits phospholipase A_2. P1 and P2 share an identical tetrapeptide (P4) which is required for inhibition, although P4 alone does not inhibit this enzyme. We found the mechanism of inhibition of platelet aggregation and secretion by the nonapeptides and P4 varied depending on whether platelets were thrombin- or ADP-activated. All three peptides decrease thrombin esterolytic activity and thereby inhibit thrombin-induced platelet activation. P1 decreases ADP-induced aggregation and serotonin secretion by inhibiting phosholipase A_2 whereas P4 decreases only aggregation by blocking fibrinogen binding to activated platelets. The P4 sequence in P1 may affect the interaction of P1 with platelets since the presence of P4 potentiates P1 inhibition of platelet activation.

Uteroglobin (UTG)[1] or Blastokinin [2] is a low molecular weight (15.8 kD) secretory protein, first discovered in rabbit uterus during early pregnancy and later found in the tracheobronchial tree, gastrointestinal tract, prostate and seminal vesicle of the rabbit. Expression of this protein is under multihormonal control. Mature uteroglobin is a dimer made up of two antiparallelly oriented monomers each of 70 identical amino acids, connected by two inter-chain disulfide bridges. An immunologically crossreactive protein similar in molecular weight to rabbit uteroglobin monomer is detectable in human tracheobronchial epithelium, prostate and progestogenic uterus.

Abbreviations: UTG-uteroglobin, PLA2-phospholipase A_2, PBS-phophate buffered saline, AA-arachidonic acid, PAF-platelet activating factor.

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Of several biological properties ascribed to rabbit uteroglobin its immunomodulatory/antiinflammatory effects appear to be the more biomedically important ones.(for a review see ref. 3).
Recently, UTG has been reported to be a potent inhibitor of platelet aggregation induced by thrombin [4]. The mechanism by which UTG exerts its observed biological activities including inhibition of platelet aggregation, is considered to be due, at least in part, to its ability to inhibit phospholipase A_2(PLA2) (E.C.3.1.1.4) . PLA2 is a key enzyme involved in production of arachidonic acid (AA) which is the substrate for synthesis of various eicosanoids (eg. prostaglandins, thromboxanes, leukotrienes, etc.), some of which may mediate platelet aggregation.[5,6] The sequence, MQMKKVLDS (P1), in UTG has been identified as an active site for inhibition of PLA2[7] but it is not known whether this nonapeptide inhibits platelets or has anticoagulant effects. A corticosteroid induced protein, lipocortin[8], is also known to inhibit PLA2 and to have anticoagulant effects[9,10], but its effect on platelets has not been studied. Lipocortin contains the sequence HDMNKVLD (P2) which is partially homologous to the nonapeptide from UTG[7]. Synthetic P1 and P2 are potent inhibitors of PLA2; but the core tetrapeptide, KVLD (P_4), which is common to both, does not have detectable PLA2-inhibitory activity [7]. In the present work we have found that thrombin-induced aggregation is weakly inhibited by P1, P2 and P4 in proportion to inhibition of thrombin esterolytic activity by these peptides. P1 and P4 are more potent inhibitors of ADP-induced platelet aggregation, by decreasing PLA2 activity in the case of P1 and by decreasing fibrinogen binding to activated platelets in the case of P4. Together P1 and P4 produce an additive inhibitory effect on aggregation and secretion.

Materials and Methods

Peptide synthesis and storage

The peptides were synthesized by an Applied Biosystems model 430A peptide synthesizer. Ninhydrin monitoring was used to determine the coupling efficiency and cleavage of peptides from the solid matrix and removal of side-chain protecting groups were achieved by an optimized hydrofluoric acid method. Peptides were purified by reverse-phase HPLC on a C_8 stationary phase and the purity was determined by HPLC and amino acid analysis. Lyophilized peptides were stored in sealed glass ampules containing oxygen-free argon or nitrogen at -20°C for 2-3 weeks without substantial loss of activity. These peptides were dissolved in desired buffer immediately before use and unused solutions were discarded since these peptides seem to lose inhibitory activity in solution. Availability of the peptides, particularly of P2, prohibited a more extensive investigation of their properties in certain experiments.

Platelet Aggregation

Whole blood was collected by venapuncture from volunteer donors and added (9:1 by volume) to 3.8% Na citrate, pH 7.0. Platelet rich plasma was obtained by centrifuging whole blood at 700 x g for 8 minutes and filtering on Sepharose 2B in divalent ion-free Tyrode's solution (pH 7.4, 0.2 % BSA). Gel filtered platelets(GFP) with counts between 200,000 and 400,000/ul were equilibrated with 2 mM Ca^{++} and 0.5 mM Mg^{++} for fifteen minutes after which 450 ul of GFP were incubated in aggregometer cuvettes at 37° C and stirred at 900 rpm with different volumes of peptides (2mg/ml) in phosphate buffered saline (PBS). Control aggregations were run simultaneously with equal volumes of PBS instead of peptides. Aggregation was initiated by addition of 50 ul of ADP (final concentration 2 X 10^{-5} M) or 10 uL of thrombin (final concentration 0.2 units/ml) and allowed to proceed until light transmission reached an asymptotic value. Inhibition was expressed as the percent of maximal light transmission in uninhibited controls. Peptide concentrations given are final concentrations in the reaction mixture. Fibrinogen was added to GFP to a final concentration of 0.2 mg/ml when aggregation was induced by ADP.

Platelet Secretion

Platelet rich plasma was incubated with C^{14}-serotonin (5 x 10^{-8} M) for 30 min at 37° C prior to filteration on a Sepharose 2B column. Serotonin secreted in the presence of 1 uM imipramine, was measured at the end of aggregation by adding 200 ul of aggregated mixture to 10 ul of 30% formaldehyde, vortexing and centrifuging at 8000 x g in a microfuge for 1 min. Fifty ul of supernatant were added to 10 ml of scintillation fluid and counted in a B-counter. Percent of secretion was calculated by dividing that obtained in the presence of peptides by control values after correction for background dpm.

Fibrinogen Binding

Human fibrinogen (Sigma, 97% clottable) was labelled with ^{125}I using insoluble lactoperoxidase/glucose oxidase method (Biorad) according to the manufacturer's instructions. Specific activity was 8 x 10^{16} cpm/mole. The resultant protein was 94% precipitable with 10% TCA and 75% clottable with thrombin. Gel filtered platelets (1.85 x 10^{8} cells) were incubated in 0.5 ml of Tyrode's buffer (2 mM Ca^{++}, 0.5 mM Mg^{++}) with varied concentrations of peptide and ^{125}I-fibrinogen (1.4 x 10^{-6} M) prior to addition of ADP (2 x 10^{-5} M) and binding was allowed to proceed for 5 minutes at room temperature [11]. At the end of incubation, 150 ul of the mixture was layered over 1.5 ml of 15% sucrose in microfuge tubes and centrifuged at 8,000 x g for 1 minute. Sucrose was suctioned off and the pellet was cut off and counted in a gamma counter. Specific binding was determined by incubating ^{125}I-fibrinogen and platelets without ADP.

Thrombin Esterolytic Activity

Thrombin activity was measured with tosyl-Gly-Pro-Arg-nitroanilide-acetate[12] (Boehringer-Mannheim), 10 μg/ml, in PBS (pH 7.4) mixed with 0.2 U/ml thrombin in the presence of varying concentrations of peptides or equal volumes of PBS. The rate of change in absorbance was measured at 405 nms.

Results

Figure 1 shows that the effects of P1(A), P2(B) and P4(C) on thrombin esterolytic activity and on thrombin-induced platelet

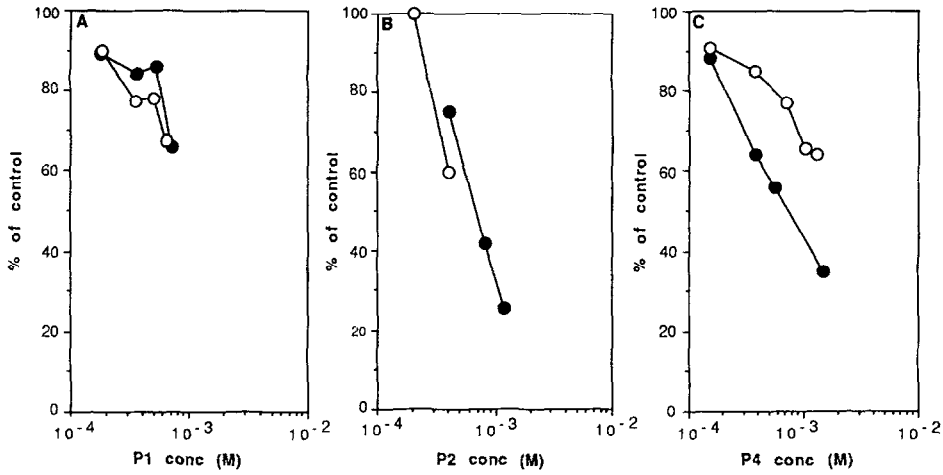

Figure 1. Inhibition of thrombin esterolytic activity and of thrombin-induced platelet aggregation by peptides P1(A), P2 (B) and P4 (C). Dark circles = esterolytic activity, clear circles = aggregation. Points are means of two separate experiments.

aggregation were roughly parallel, with P4 appearing to inhibit thrombin esterolytic activity more effectively than aggregation. As shown in Figure 2, P1 and P4 inhibit aggregation more effectively when ADP is the agonist compared to thrombin (Figure 2). The IC50 with P1 (clear circles) is 2 x 10^{-4} M and with P4 (dark circles) is 4.5 x 10^{-4} M. Since P1 is known to inhibit PLA2 it was of interest to determine whether aggregation induced by AA is inhibitable with P1. As is shown in Table 1 , when ADP is the

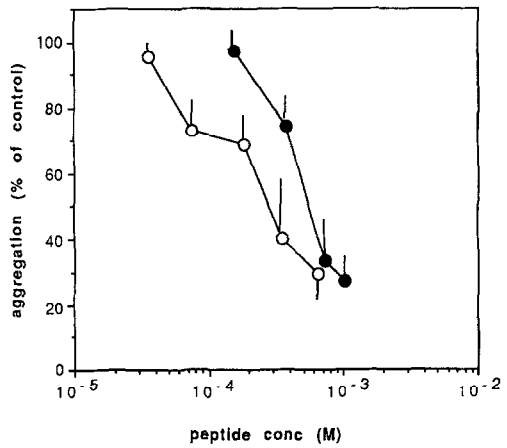

Figure 2. Inhibition of platelet aggregation induced by ADP (2 x 10^{-5} M) by P1(clear symbols) and P4(dark symbols). Percent of control aggregation was calculated by comparing maximal light transmission of platelet solutions with and without peptides. Peptide concentrations were corrected for any changes in volume. Points are means ± S.E. of three separate experiments.

Table 1. Effects of P_1 (4.5×10^{-4} M) on aggregation and secretion induced by ADP (2×10^{-5} M) or Arachidonic acid (1×10^{-4} M)

	ADP		AA	
	Aggregation	Secretion	Aggregation	Secretion
P1 (450 uM)	23.3	17.1	0	6.3

agonist, the concentration of P1 that inhibits aggregation 23.3% and secretion 17.1% does not affect aggregation and inhibits secretion only 6.3% when AA is the agonist.

P4 inhibits ADP-induced platelet aggregation even though it does not have anti-PLA2 activity in vitro[7]. This inhibition is aggregation-specific since simultaneously measured serotonin secretion was not decreased (Figure 3). P4 also inhibits ^{125}I-fibrinogen binding to ADP-activated platelets over the same concentration range (IC50 6.5×10^{-4} M) that inhibits aggregation (IC50 5.5×10^{-4}M) (Figure 4A). Fibrinogen binding by ADP-activated platelets is not inhibited by P1 (Figure 4B). Table 2 shows that P1 and P4 are additive in their inhibition of ADP-induced platelet aggregation and secretion.

Discussion

Uteroglobin (UTG) was previously reported to inhibit thrombin induced platelet aggregation through a mechanism postulated to

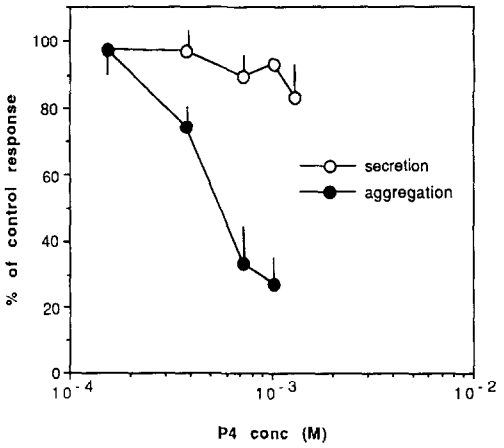

Figure 3. Effect of P4 on platelet aggregation and C^{14}-serotonin secretion. Clear circles = serotonin secretion, Dark circles = aggregation. Aggregation in the presence of peptides is expressed as percent of control values in absence of peptides. Points are the means ± S.E. of three experiments.

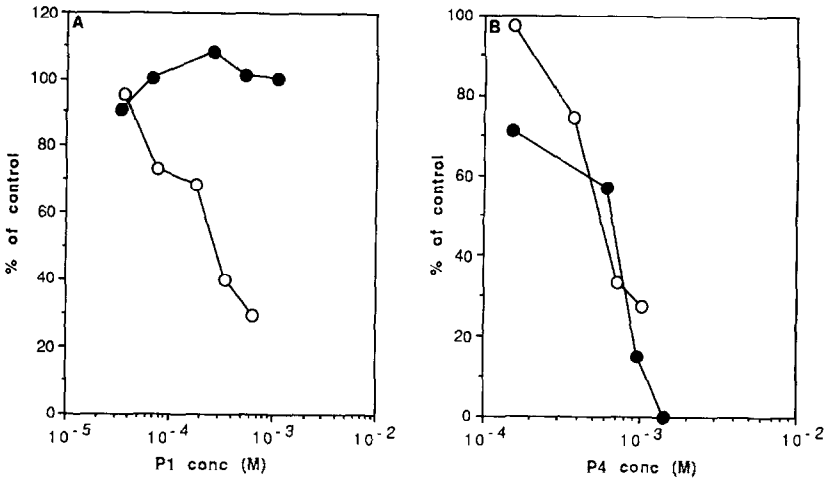

Figure 4. Effect of peptides P1 (A) and P4 (B) on platelet ^{125}I-fibrinogen binding and aggregation to ADP activated platelets. Clear circles = aggregation, Dark circles = binding. Points are expressed as percent of fibrinogen binding in absence of peptides and are the means of two experiments.

involve inhibition of PLA2[4]. The anti-PLA_2 activity of UTG has been localized to nonapeptide (P_1) which is partially homologous to an active nonapeptide (P_2) of another PLA2-inhibiting protein, lipocortin[7]. We have found that P1 and P2 are weak inhibitors of thrombin-induced platelet aggregation (IC50 of $>10^{-3}$ M) compared to UTG (IC50 of 1.4×10^{-5} M)[4]. The possibility that these nonapeptides act by a mechanism different from PLA2 inhibition was suggested by finding that both P1 and P2 decrease thrombin esterolytic activity in proportion to the degree of inhibition of thrombin-induced platelet aggregation (Figure 2A and 2B). Thrombin esterolytic activity is known to be necessary for activation of platelets[14]. Others have shown that inhibition of thrombin-induced platelet activation by biologically active

Table 2. Combined effects of P1(4.5×10^{-4}M) and P4(9.1×10^{-5}M) inhibition on platelet aggregation and secretion

	% Inhibition of ADP-induced Platelet Activation	
	Aggregation	Secretion
P1 (450 uM)	23.3	17.1
P4 (91 uM)	2.3	0

peptides such as leupeptin, is due to direct inhibition of thrombin[15].

The quantitated degree of inhibition of platelet PLA2 cannot be quantitated by the degree of inhibition of thrombin-activated platelets since thrombin activation involves multiple mechanisms. For example, activities dependent on phospholipase C or flux through Ca^{++} channels [16,17] can compensate for a block in an AA pathway. ADP activation of platelets is more sensitive to inhibition by P1 (IC50 2 x 10^{-4} M) most likely because of its greater dependence on AA mediated pathways that are mediated by PLA2. Finding that activation of platelets by exogenous AA is not inhibited by P1 is consistent with the concept that decreased ADP-activation by P1 is caused by inhibition of PLA2. Platelet aggregation is less sensitive to inhibition by P1 than is the activity of purified PLA2 (IC50 ~10^{-9})[7]. This difference may reflect either poor access by P1 to the platelet membrane enzyme or intrinsic differences between platelet PLA2 and the porcine PLA2 enzyme used in the assay. P1 has been shown to decrease localized edema and inflammation induced by carageenan[7] and also to decrease platelet activating factor (PAF) production in isolated macrophages.[18]. Since PLA2 catalyzes the reaction to produce the immediate precursor of PAF, lyso-PAF, from 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine[19,20], P1 can decrease production of PAF by inhibiting PLA2. These responses indicate that P1 is capable of affecting this enzyme in other cell types and suggest that its access to the platelet PLA2 may be limited.

P1 and P2 share an identical sequence, P4, which is necessary for their PLA2 inhibitory activity but by itself does not inhibit this enzyme[7]. Nevertheless, P4 decreased ADP-induced platelet aggregation but not platelet secretion (Figure 3). Aggregation is a separate response from secretion that depends on fibrinogen binding to a plasma membrane receptor, glycoprotein IIb/IIIa, which is expressed upon platelet activation[13,22]. P4 inhibited ^{125}I-fibrinogen binding with an IC50 of 6.5 X 10^{-4} M which is very similar to the IC50 of 4.5 x 10^{-4} M for inhibition of platelet aggregation by P4(Figure 4). Fibrinogen binding to activated platelets is through two amino acid sequences: Arg-Gly-Asp-Ser (RGDS), a recognition sequence common to many attachment proteins[22], and by a decapeptide sequence HLGGAKQAGDV found in

the COOH terminal of the fibrinogen A chain[23]. Presence of either one of these as free peptides can prevent fibrinogen binding to activated platelets and block platelet aggregation. RGDS has an IC50 of 9 X 10^{-6} M while the COOH terminal sequence has been reported to have an IC50 of 1.7 x 10^{-5} M in ADP-induced platelet aggregation[23]. P4 (KVLD) resembles RGDS by having a positive and a negative charge in close proximity, separated by one amino acid in RGDS and two amino acids in KVLD. Further evidence that KVLD can interact with RGDS receptors is the finding that replacement of arginine in RGDS by lysine to give KGDS, results in a peptide that inhibits fibrinogen binding as effectively as KVLD[24]. P1, which contains the P4 sequence, did not inhibit fibrinogen binding most likely because steric hindrance by the additional amino acids prevented P1 binding to the fibrinogen receptor. Similar observations have been reported with the RGDS sequence incorporated into longer peptides where adjacent amino acids alter its ability to inhibit fibrinogen binding[18].

Thrombin- and ADP-induced platelet aggregation were not equally inhibited by P4, a finding that may be attributable to differences in fibrinogen requirements for ADP- and thrombin-induced aggregation. Fibrinogen released by thrombin from platelet alpha granules may not be as readily available for aggregation as the exogenous fibrinogen present when ADP is the agonist[25].

In summary, peptides obtained from UTG are less active in inhibiting thrombin-induced platelet aggregation than intact UTG and act by directly decreasing thrombin enzymatic activity. This inhibitory mechanism differs from the anti-PLA2 activity of the parent molecule. The conserved sequence P4, found in the nonapeptides P1 and P2, inhibits fibrinogen binding to ADP-activated platelets although P1 does not have this activity. P4 does not inhibit PLA2 but potentiates the platelet-inhibitory effects of P1(Table 2), possibly by increasing the availability of P1 by displacing it from inactive platelet sites to which P1 may be bound by its P4 sequence.

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