Isolation of an Inhibitor Selective for Collagen-stimulated Platelet Aggregation from the Soft Tick *Ornithodoros moubata*

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Soluble extracts from the soft tick *Ornithodoros moubata* were found to inhibit collagen-, ADP-, and thrombin-stimulated platelet aggregation. One inhibitory component was purified to homogeneity by a combination of gel filtration, ion-exchange, and reverse phase high pressure liquid chromatography. The purified activity, named moubatin, is a protein of molecular weight 17,000 and it inhibits the aggregation of washed human platelets stimulated by collagen with an IC₅₀ of ~50 nM in the standard assay. At a concentration of moubatin that maximally inhibited collagen-stimulated platelet aggregation, no inhibition of aggregation initiated by other effectors, including arachidonic acid, thrombin, ristocetin, and the calcium ionophore A23187, was observed. Moubatin also inhibits collagen-dependent aggregation in plasma. At a higher concentration of moubatin (>1 µM) it was also possible to demonstrate an inhibitory effect on the final extent of aggregation induced by a low concentration of ADP. Although moubatin selectively inhibits platelet activation by collagen, it has only a minimal effect on the adhesion of platelets to collagen. The amino acid sequences of peptides derived from proteolytic cleavage of moubatin suggest that moubatin is a unique protein, consistent with its novel functional activity.

A wide variety of organisms obtain nourishment by sucking blood. Although the mechanism by which each carries out this process varies considerably, in most instances the predator is faced with the formidable task of maintaining the flow of blood from the tissue or blood vessel of the victim. A broad spectrum of different types of inhibitors of normal hemostasis have been found in nature, including anticoagulants, anti-thrombotics, and thrombolytics. Frequently these are found in the salivary glands of blood sucking creatures. For example, saliva of the leech *Hirudo medicinalis* contains hirudin (1), a potent inhibitor of thrombin, a key enzyme in the coagulation pathway as well as a potent activator of platelets. A platelet fibrinogen receptor antagonist was recently found in the saliva of another leech, *Macrobdella decora* (2). The saliva of the vampire bat contains the third type of inhibitor described above, a plasminogen activator (3). This enzyme is capable of catalyzing the dissolution of a clot.

Other less well characterized factors in the saliva of these organisms have been described which may also play a role in facilitating the flow of blood. These include nonspecific anti-

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EXPERIMENTAL PROCEDURES

Materials—Equine tendon type I collagen, arachidonic acid, ADP, and ristocetin were from Chrono-Log Corp., Havertown, PA. Thrombin was from American Diagnostica; human fibrinogen and A23187 were from Calbiochem; Endopeptidase Lys-C was purchased from Boehringer. All other reagents were from Fisher and Sigma.

Platelet Isolation and Functional Activity Assays—Platelets were isolated from healthy human volunteers free from aspirin and other drugs for at least 8 days. Blood was anticoagulated with acid citrate dextrose and the platelets washed and isolated as described previously (7). The final platelet suspension was in a modified Tyrode's buffer without Ca²⁺ (5 mM Heps, 3 mM KCl, 134 mM NaCl, 0.3 mM Na₂HPO₄, 2 mM MgCl₂, 5 mM glucose, 12 mM NaHCO₃, 3.5 mg/ml bovine serum albumin at pH 7.4) adjusted to 3 × 10⁵ platelets/ml after counting in a model 2M Coulter Counter (Coulter Electronics, Hialeah, FL). For studies in plasma, the blood was collected in 0.38% citrate and the platelet-rich plasma obtained by differential centrifugation.

Aggregation of the washed platelets was used to monitor platelet inhibitory activity in the tick soluble extracts and for each of the fractions tested throughout the purification of moubatin. The tick extract, column fraction, or aliquot of purified protein was preincubated with 0.2 or 0.25 ml of platelets at 37 °C with 0.2 mg/ml human fibrinogen for 2 min. Collagen at 1 or 2 µg/ml, or another agonist, was added, and the aggregation was monitored on a Sienco or a Chronolog aggregometer. The percentage inhibition was calculated based on the final extent of aggregation of the test samples compared with the appropriate buffer control. Similar results were obtained when the rate of aggregation of the test samples compared with controls was monitored.

Analytical Techniques—SDS-PAGE under reducing conditions was run on 20% acrylamide Phast Gels (Pharmacia Phast Gel System) and proteins were detected by staining with silver according to the protocol of the manufacturer. Protein was estimated using the bicinchoninic acid (BCA) assay (Fierce Chemical Co.) with bovine serum albumin as the standard.

Purification of Moubatin—O. moubata ticks were obtained frozen

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; BiTris, 2-[bis(2-hydroxyethyl)aminio]-2-(hydroxyethyl)-propane-1,3-diol; HPLC, high pressure liquid chromatography.
from Antibody Associates, Ltd. (Bedford, TX) and stored at -70 °C until used. A typical preparation was initiated with 400-500 whole ticks containing about 12 g of material. The ticks were disrupted in batches of 50 with a Polytron homogenizer (Brinkmann Instruments) in 10 ml of 25 mM Hepes buffer, pH 7.0, containing 0.15 M NaCl and the protease inhibitors E-64, pepstatin, chymostatin, and leupeptin (50 μM each). The homogenate was centrifuged at 100,000 × g for 20 min, and the resulting pellets were re-extracted in 5 ml of the same buffer and recentrifuged. The combined supernatants (approximately 800 mg of protein) were tested for inhibitory activities and used for the purification of moubatin.

The crude soluble extract was diluted 3-fold with water to lower the ionic strength and applied to a 25-ml Fast Q Column equilibrated in 25 mM BisTris-HCl, pH 7.0. The column was washed with the same buffer until the absorbance at 280 nm decreased to less than 0.05. Bound proteins were eluted with 0.8 M NaCl (100 ml) in the same buffer, dialyzed against water to remove the salt, and lyophilized in the presence of 0.1 mg/ml sucrose. The lyophilized material was redissolved in H2O and applied to a column of Sephadex G-50 (Superfine, 2.5 × 50 cm) equilibrated in 50 mM Hepes, pH 7.0.

The peak fractions containing platelet aggregation inhibitory activity were pooled and applied to a Pharmacia Mono Q anion-exchange column (1 × 10 cm) equilibrated in 20 mM BisTris-HCl, pH 6.0, controlled by a fast protein liquid chromatography system. The bound proteins were eluted with a gradient of NaCl (0–0.3 M) in the same buffer. Inhibitory activity eluted in two peaks at 0.12 and 0.25 M NaCl. The active fractions in each peak were pooled separately and concentrated by lyophilization. The dried material was redissolved in 0.15 M Na2HPO4, 0.1% acetic acid. Fractions of 0.5 ml were collected, and aliquots (2–10 μl) were assayed for their ability to inhibit the aggregation of platelets in the presence of collagen ( ), as described under “Experimental Procedures.” Assays for the inhibition of coagulation factor Xa ( ) and thrombin ( ) were carried out as described previously (9).

**Results**

**Characterization of Tick Extract for Inhibitory Activities**

Soluble crude extracts of *O. moubata* ticks were prepared as described under “Experimental Procedures” and concentrated by lyophilization in the presence of sucrose. The dried material was redissolved in H2O and fractionated by gel filtration on Sephadex G-50. The fractions were assayed for the presence of inhibitors of blood coagulation enzymes. Using chromogenic substrates and purified proteolytic enzymes from the coagulation cascade, inhibitors of factor Xa and thrombin were identified (Fig. 1). The inhibitor of factor Xa, TAP, has been purified to homogeneity (9), and its properties have been investigated in detail (10–13). Since platelet activation is also essential for clot formation and antplatelet activities had been reported in hard ticks (14), we tested whether the gel filtration column fractions of the soluble extract from these soft ticks also contained platelet aggregation inhibitors.

Aggregation dependent on collagen and thrombin was blocked by fractions from the gel filtration column. The activity that blocked thrombin-induced aggregation co-eluted with an activity that also inhibited the hydrolysis of a chromogenic substrate by thrombin. Further characterization of this activity showed that it was a thrombin inhibitor (data not shown), and it was not pursued further. The inhibitor of collagen-dependent aggregation eluted in a single peak with an apparent molecular weight of ~20,000 (Fig. 1). This activity was taken for further purification.

**Purification of Moubatin**

The active fractions with platelet aggregation inhibitory activity from the gel filtration column were pooled and applied to an anion-exchange column (Mono Q), and the bound protein was eluted with a gradient of NaCl. This step resolved two peaks of inhibitory activity eluting at ~0.125 and 0.25 M NaCl (Fig. 2). Each of these two peaks was then purified separately.

The pools of each of the peak inhibitory fractions from the Mono Q chromatography step were lyophilized and then chromatographed on Sephadex G-75. Aggregation inhibitory activity eluted in a single peak for each sample with the same apparent molecular mass (~20 kDa) (data not shown). After gel filtration, only the activity that eluted at 0.125 M NaCl...
from Mono Q was purified further because of insufficient material remaining in the other sample pool. The G-75 pooled material was subjected to two additional purification steps consisting of repeated reverse phase chromatography on a Pro RPC column. Figs. 3 and 4 show that after chromatography on the second reverse phase column, the absorbance at 214 nm and the activity co-eluted. SDS-PAGE of this material (Fig. 5) showed a single band (mass, \( \sim 17 \) kDa) when stained with silver. We have named this inhibitor of platelet aggregation "moubatin," since it was isolated from the tick \textit{O. moubata}. Approximately 100 \( \mu \)g of purified protein was obtained from a typical preparation.

**Isolation of Peptides**—To obtain information on the primary structure of moubatin, the purified polypeptide was submitted to automated sequence analysis. The yield of amino acids by this procedure from \( \sim 50 \) pmol of the purified polypeptide was less than 10% of the expected amount, suggesting that the protein was blocked at the amino terminus. Consequently, the reduced and alkylated polypeptide was digested with the protease Lys-C, which cleaves after lysyl residues. The resulting peptide fragments were then resolved by reverse phase HPLC (Fig. 6). Each of the peaks was sequenced and seven unique peptides were obtained as shown in Table I. Several of these sequences were confirmed by analysis of peptides derived from a cyanogen bromide digest of moubatin. The sequences of these peptides did not show any significant homology to collagen or any other protein in the Swiss PROT data base. Overall, the sequences correspond to 102 amino acid residues, which, based on the molecular weight of moubatin, must represent more than 50% of the total number of amino acids in the polypeptide.

**Properties of Purified Moubatin**—An example of the actual data obtained in experiments which examined the effect of moubatin on collagen-stimulated platelet aggregation is shown in Fig. 7A. Moubatin blocked both the rate and extent of aggregation, and at the highest concentration examined, all aggregation and shape change were completely inhibited. From the concentration dependence of the inhibition of ag-
partially inhibited the final extent of aggregation induced by moubatin shown in Fig. 7B. The percent control extent of aggregation with increasing concentration of moubatin in B. The results are the mean ± S.E. from three separate experiments.

Moubatin also inhibited collagen-dependent aggregation of platelets, including several monoclonal antibodies and collagen-derived peptides, have been shown to be useful tools to study this process, but their use has been limited by strictly defined conditions required to demonstrate their inhibitory activity (16-20).

The 17-kDa polypeptide that we have isolated from ticks, which is named moubatin, appears to be a selective high affinity inhibitor of collagen-dependent platelet aggregation. Using purified platelets aggregation induced by a variety of agonists is not inhibited by moubatin at a concentration that completely inhibits 1 µg/ml collagen-stimulated aggregation. Moubatin also inhibited collagen-dependent aggregation of platelets in plasma. At concentrations at least 10-fold higher than that required to block collagen-stimulated aggregation, moubatin did inhibit the second phase of platelet aggregation induced by ADP at concentrations ≤5 µM. This partial inhibitory effect was not observed with LAPP (7) and warrants further investigation. However, because this inhibitory effect is observed only at higher concentrations of moubatin, these studies cannot be carried out with native material which is available in limited quantities. We have therefore cloned and expressed this protein in large amounts, and the results of these and related investigations are presented in the accompanying paper (21).

Moubatin displays unique functional activity. Unlike the other natural product protein inhibitors of collagen stimulation of platelets, including LAPP (7), calin (6), and the B. atrox snake venom protein (15), moubatin has no significant effect on platelet adhesion to collagen in static adhesion assays (data not shown).
FIG. 8. Effect of moubatin on platelet aggregation in the presence of different agonists. Washed platelets were incubated with moubatin and aggregation monitored after agonist addition as described in the legend to Fig. 7. The downward deflection of the tracing indicates the time of agonist addition. The agonist and moubatin concentrations were the following: 2 µg/ml collagen, 92 nM moubatin; 20 µM ADP, 92 nM moubatin; 0.25 mM arachidonic acid, 92 nM moubatin; 0.4 nM thrombin, 92 nM moubatin; 1.25 mg/ml ristocetin, 940 nM moubatin. The results are representative of three to six separate experiments.

| Stimulus          | [Moubatin] (µM) | % inhibition |
|-------------------|-----------------|--------------|
| Collagen (1 µg/ml)| 0.09            | 100          |
| Collagen (1 µg/ml)| 1               | ND*          |
| ADP 2 µM         | ND*             | 60           |
| ADP 20 µM        | 0               | 40           |

* ND, not determined.

TABLE II

Effect of moubatin on platelet aggregation in plasma

Platelet-rich plasma (0.25 ml) was incubated with the indicated concentration of moubatin at 37 °C for 2 min followed by addition of agonist and aggregation was monitored as described under "Experimental Procedures." Results are expressed as the percent inhibition of the final extent of aggregation. The results shown are from one experiment representative of three separate experiments using plasma from different donors.

REFERENCES

1. Markwardt, F. (1970) Methods Enzymol. 19, 925-932
2. Seymour, J. L., Henzel, W. J., Nevins, B., Stults, J. T., and Lazarus, R. A. (1990) J. Biol. Chem. 265, 10143-10147
3. Gardell, S. J., Dong, L. T., Diehl, R. E., York, J. D., Hare, T., Register, R. B., Jacobs, J. W., Dizon, R. A., and Friedman, P. A. (1989) J. Biol. Chem. 264, 17947-17952
4. Righi, M., Levy, H., Eldor, A., Iizuka, H., Dizengoff, M., Orevi, M., Horovitz, A., and Galun, R. (1987) Comp. Biochem. Physiol. 88C, 95-98
5. Righi, M., Levy, H., Iizuka, H., Dizengoff, M., Orevi, M., Ajourkis, A., Horovitz, A., and Galun, R. (1987) Comp. Biochem. Physiol. 87B, 567-572
6. Munro, R., Powell Jones, C., and Sawyer, R. T. (1991) Blood Coag. Fibrinol. 2, 179-184
7. Connolly, T. M., Jacobs, J. W., and Condra, C. (1992) J. Biol. Chem. 267, 6833-6838
8. Keller, P. M., Schultz, L. D., Condra, C., Karczewski, J., and Connolly, T. M. (1992) J. Biol. Chem. 267, 6886-6904
9. Waxman, L., Smith, D. E., Arca, R. E., and Vlasuk, G. P. (1990) Science 248, 593-596
10. Jordan, S. P., Waxman, L., Smith, D. E., and Vlasuk, G. P. (1990) Biochemistry 29, 11085-11090
11. Sardana, M., Sardana, V., Rodkey, J., Wood, T., Ng, A., Vlasuk, G. P., and Waxman, L. (1991) J. Biol. Chem. 266, 3560-3565
12. Schaffer, L. W., Davidson, J. T., Vlasuk, G. P., and Siegl, P. K. S. (1991) Circulation 84, 1714-1748
13. Vlasuk, G. P., Ramjit, D., Fujita, T., Dunwiddie, C. T., Natt, E. M., Smith, D. E., and Shebski, R. J. (1991) J. Biol. Chem. 266, 257-262
14. Ribeiro, J. M. C., Makoul, G. T., Levine, J. Robinson, D. R., and Spielman, A. (1986) J. Exp. Med. 161, 322-344
15. Smith, J. B., Dangelmier, C., and Selak, M. (1991) FEBS Lett. 283, 307-315
16. Kunicki, T., Nugent, D. J., Staats, S. J., Orbeckowski, R. P., Wayner, E. A., and Carter, W. G. (1989) J. Biol. Chem. 264, 4516-4519
17. Coller, B. S., Beer, J. H., Scudder, L. E., and Steinberg, M. H. (1989) Blood 74, 152-192
18. Staatz, W. D., Rajpara, S. M., Wayner, E. A., Carter, W. G., and Santoro, S. A. (1989) J. Cell Biol. 108, 1917-1924
19. Staatz, W. D., Fok, K. F., Zetter, M. M., Adams, S. P., Rodriguez, B. A., and Santoro, S. A. (1991) J. Biol. Chem. 266, 7363-7366
20. Legrand, Y., Margirian, A., Franchi, P., Faure, F., and Caen, J. P. (1980) Biochem. Biophys. Res. Commun. 96, 1373-1378
21. Keiler, P. W., Waxman, L., Arnold, B. A., Schultz, L. D., Condra, C., and Connolly, T. M. (1993) J. Biol. Chem. 268, 5432-5436
22. Russhadi, B., and Fiersschbach, M. D. (1987) Science 238, 491-497
23. Gould, R. J., Polokoff, M. A., Friedman, P. A., Huang, T.-F., Holt, J. C., Cook, J. A., and Niewiarowski, S. (1990) Proc. Soc. Exp. Biol. Med. 185, 168-173
24. Santoro, S. A. (1986) Cell 46, 913-920

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2 T. M. Connolly, L. Waxman, and J. Karczewski, manuscript in preparation.

Collagen ADP Arachidonic Acid Platelet-rich plasma

Thrombin Ristocetin A23187

Light Transmission

1 min

5449