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In vivo metabolism of reversibly inhibited α-thrombin*

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Pharmacologic intervention for thrombotic disorders has been greatly restricted by the paucity of safe, effective drugs. The recent development of reversible thrombin inhibitors offers a novel approach for therapy [1–3]. These drugs, which are usually derivatives of arginine or benzamidine, competitively inhibit thrombin by binding at the active site. In experimental animals, these inhibitors prevent disseminated intravascular coagulation and reduce arterial thrombus size [4, 5].

In vivo, the major inhibitor of thrombin is antithrombin III (AT III) which binds tightly to the active site of thrombin [6]. The At III–thrombin complexes (TAT) are then rapidly cleared from the circulation by a recently described specific receptor located on hepatocytes [7]. Free thrombin can also bind to an endothelial receptor before its interaction with AT III [8]. When thrombin is treated with diisopropyl fluorophosphate, the diisopropyl-thrombin (DIP-thrombin) also clears on the thrombin receptor, indicating that protease activity is not required for thrombin clearance [7, 8]. Autopsy studies demonstrate that free thrombin and DIP-thrombin clear primarily in lung when injected intravenously [7, 8].

In view of the extensive biochemical and pathological studies with reversible thrombin inhibitors, the present investigation was undertaken to study the metabolism of reversibly inhibited thrombin and its relationship to the normal catabolic pathways.

Methods

Reagents. α-Thrombin was purified from Cohn fraction II/III (a gift of Dr. Wayne Wescott and Alpha Therapeutics) by the method of Fenton *et al.* [9]. The activity was 2700 units/mg. The reversible synthetic thrombin inhibitor compound No. 805 (MCI-9038, MD-805)† was a gift of Mitsubishi Chemical Industries Ltd., Tokyo, Japan. The compound was dissolved in water to 1 mg/ml as recommended by the manufacturer. DIP-thrombin, α-thrombin, α_2-macroglobulin-methylamine, AT III and purified TAT were prepared as previously described [7, 10, 11]. Proteins were radioiodinated by the method of David and Reisfeld [12]. Radiolabeled α-thrombin retained greater than 90% clotting activity.

Clearance studies. Clearance studies were performed in female CD 1 mice as previously described [7]. Briefly, approximately 2.5 μg of radiolabeled protein was injected into the tail vein, and 25 μl samples were obtained from the retroorbital venous plexus at various time intervals beginning at 5 sec. These samples were precipitated in 5% ice-cold trichloroacetic acid, and the precipitates were counted in a gamma counter. Competition studies were performed by injecting approximately 1000-fold molar excess of unlabeled ligand with the radiolabeled ligand. Tissue distribution studies were performed by killing five animals at both 2 min and 20 min after injection and counting the tissues.

SDS–gel electrophoresis of blood. Polyacrylamide gel electrophoresis of blood treated with sodium dodecyl sulfacte (SDS) and β-mercaptoethanol was utilized to determine the fate of thrombin injected into the mouse circulation. To perform these studies, 4 μg of radiolabeled thrombin treated with 20 μg of compound No. 805 was injected into the mouse circulation as described above. Duplicate 25 μl blood samples were drawn at each time point, one for determining radioactivity and one for electrophoretic analysis. The latter samples were treated with an equal volume of sample buffer containing β-mercaptoethanol and electrophoresed utilizing a Tris–borate–SDS gel system at pH 8.5 (7.5% gels). The resultant gel was stained and destained in the usual manner, and each gel lane was sliced into 2 mm strips for counting in a gamma counter. TAT and AT III were employed as markers in separate gel lanes.

Results

The clearance of α-thrombin injected with various doses of compound No. 805 is shown in Fig. 1. These curves show a peculiar peak in counts which occurs after the initial rapid falloff. The time of appearance of this peak was dose dependent and shifted to the right with increasing dosage of the drug.

The natures of the initial falloff and subsequent peak were investigated by competition studies. When labeled thrombin and a 1000-fold molar excess of DIP-thrombin were injected with 50 μg of inhibitor, the peak was not seen and the early rapid phase was blunted (Fig. 2). The label disappeared in a manner similar to that previously reported for TAT complexes [7]. The blunting of the early rapid disappearance phase by competing DIP-thrombin implies that this phase was mediated by binding to the endothelial

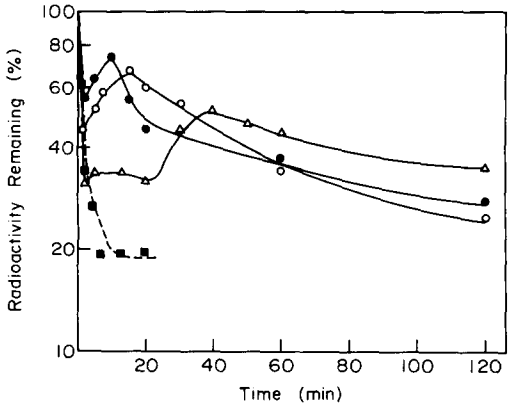

Fig. 1. Clearance of α-thrombin administered with compound No. 805. Clearance studies were performed in mice as described in 'Methods'. The data from four mice were averaged. The variation at each point was generally under 10%. The drug concentrations administered with the α-thrombin sample were: 10 μg (●), 20 μg (○), and 100 μg (△). The clearance of DIP-thrombin is shown for comparison (■).

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† Compound No. 805 is (2*R*,4*R*)-4-methyl-1-[N^2-(3-methyl-1,2,3,4-tetrahydro-8-quinoline sulfonyl)-L-arginyl]-2-piperidinecarboxylic acid monohydrate.

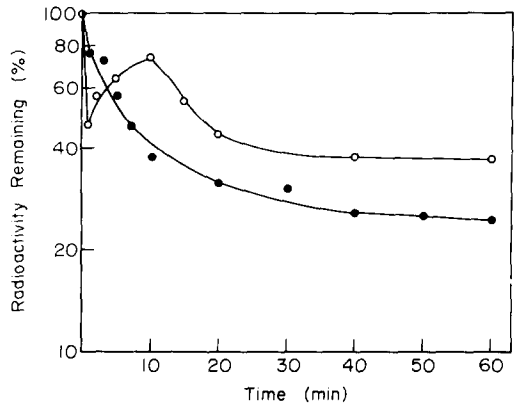

Fig. 2. Clearance of thrombin administered with 50 μg of compound No. 805 in the presence of DIP-thrombin. A 1000-fold molar excess of unlabeled DIP-thrombin (●) was utilized in these competition experiments. The clearance of drug-inhibited thrombin is shown for comparison (○).

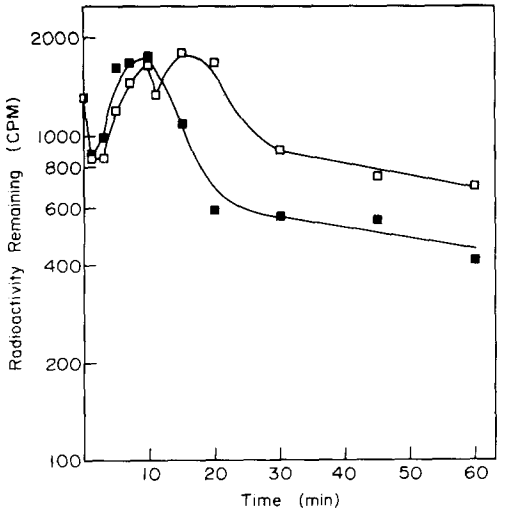

Fig. 3. Clearance of thrombin administered with 50 μg of compound No. 805 in the presence of TAT. A 1000-fold molar excess of unlabelled TAT (□) was utilized in these experiments since previous studies indicate that a large molar excess of unlabeled TAT is required to adequately compete for the clearance of radiolabeled TAT [7]. The clearance of thrombin in the presence of drug is shown for comparison (■).

thrombin receptor and suggests that the peak resulted from release of labeled thrombin from the endothelium into the circulation. This thrombin may have been free or more likely complexed with plasma thrombin inhibitors, particularly AT III.

The formation of TAT was, therefore, investigated by competition with a 1000-fold molar excess of preformed, unlabeled TAT at the time of the peak (Fig. 3). This study showed effective competition of TAT with the tracer, thus confirming the formation of TAT from the [^{125}I]thrombin.

* Compound No. 805 is known to have a half-life of about 5 min in the rat; Dr. Y. Tamao, personal communication.

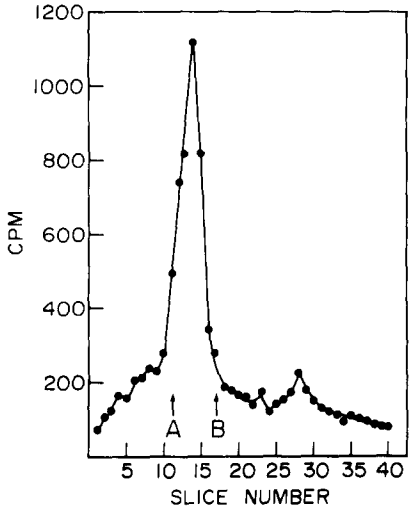

Fig. 4. SDS–polyacrylamide gel electrophoresis of blood from an animal receiving α-thrombin with compound No. 805. The study was performed as described in 'Methods'. The distribution of counts at 20 min after injection is shown in the figure. The arrows indicate the positions of TAT (A) and AT III (B), utilized as markers.

Tissue distribution studies were also consistent with early binding of drug-inhibited thrombin to the lung and transfer to the liver. When autopsies were performed 2 min after injecting drug–thrombin complexes, 61 ± 4% (S.D.) of the counts was found in the lungs and 26 ± 4% (S.D.) in liver but, when animals were killed after 20 min, 61 ± 6% (S.D.) of the counts was in liver and 24 ± 6% (S.D.) in the lungs.

The above studies are consistent with ultimate clearance of the α-thrombin as the TAT complex. In order to directly address this question, blood samples were obtained throughout the time course of clearance and subjected to SDS–polyacrylamide gel electrophoresis (Fig. 4). These studies demonstrate that at least 80% of the counts was present in the region where the TAT complex migrates. There is a small shoulder (less than 10%) on the left in a region which may correspond to the position of reduced α_2-macroglobulin–protease complexes [11], and there was also a small peak of free thrombin. To demonstrate by competition studies that some α-thrombin is catabolized as an α_2-macroglobulin complex, drug–α-thrombin complex was injected into mice, and a 1000-fold molar excess of α_2-macroglobulin-methylamine was then injected at the time of the peak (Fig. 3). There was a slight competitive effect noted (data not shown) by this complex which clears after binding to the reticuloendothelial α_2-macroglobulin–protease receptor [11].

Discussion

The above studies indicate a complicated clearance for reversibly inhibited thrombin. The initial phase can be attributed to binding of thrombin to endothelial receptors in lung [8]. This was demonstrated by the effective competition with DIP-thrombin (Fig. 2) which is known to bind to the endothelial receptor [7, 8]. Tissue distribution studies show early uptake by lung which is a rich reservoir for the endothelial receptor [8].

The second phase of clearance of thrombin involves the interaction of the thrombin with AT III and subsequent clearance. This occurs as the circulating level of the drug is falling*, thus freeing the thrombin active site for interaction with AT III. This interaction probably occurs on the endothelial surface thus releasing TAT complexes into the circulation, but interaction with free thrombin cannot be

excluded. The dose–respose experiments are consistent with this hypothesis as the peak shifted progressively to the right with higher doses of the inhibitor. This indicates that less free thrombin was available early in the time course when higher doses were administered. Previous studies of the metabolism of free thrombin in the rabbit also suggest that thrombin reacts with AT III on the vessel wall [8]. The reaction appears to occur here because heparinoids present on the vessel wall are thought to greatly accelerate the rate of reaction between AT III and thrombin [8].

The ultimate fate of most of the drug-inhibited thrombin involves the formation of the TAT complex which clears in the liver [7]. This was demonstrated by the effective blockade of the clearance when unlabelled TAT was injected at the time of the peak and by SDS–polyacrylamide gel electrophoresis of blood samples obtained after injecting drug–thrombin complexes (Figs. 3 and 4). The shift in counts from the lung to the liver is also indicative of this pathway. The data presented in Fig. 3 are not plotted in the typical manner utilized in clearance studies; namely, as percent of the ligand remaining in the circulation (see Ref. 7 for a review of the mouse clearance model). The data in Fig. 3 were plotted as counts per minute in the circulation because the level of thrombin reappearing, as judged from the raw data, actually exceeded the initial number of counts recorded when the blood was drawn from the retroorbital venous plexus at 5 sec. This phenomenon is an artifact of this sampling procedure. Thrombin clearance in the lung is extremely rapid [7, 8]; hence, the blood drawn from the retroorbital venous plexus already will have lost a fraction of the thrombin as the blood makes its first pass through the lung. Thus, the reappearance peak may show higher counts than the initial blood sample.

The clearance of reversibly inhibited thrombin has obvious clinical implications. Since the clearance of thrombin is primarily dependent on AT III, careful monitoring of both the drug and AT III levels appears imperative especially when the drug is being discontinued. If AT III levels were suboptimal, AT III concentrates could be administered to ensure adequate inhibitory capacity. Since, however, the amount of thrombin generated in thrombotic conditions is usually quite small, adequate AT III levels should not be a major obstacle for the use of these drugs in most situations. In this regard, compound No. 805 has been reported to be an effective therapy for preventing thrombosis in AT III deficient rats [13].

In summary, reversibly inhibited thrombin is removed from the circulation in two steps, binding to endothelial cells primarily in lung followed by clearance in the liver as the TAT complex.

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[^{3}H]Haloperidol binding to more than one site in rat brain striatum

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In order to re-examine the suitability of [^{3}H]haloperidol as a selective ^{3}H ligand for D_2 dopamine receptors [1], we tested the properties of [^{3}H]haloperidol binding to rat brain striatum. The D_2 receptor is defined as that dopaminergic site with high affinity (nanomolar) for neuroleptics and low affinity (micromolar) for dopamine agonists [2].

This report demonstrates that [^{3}H]haloperidol binds to more than one population of sites, when 0.1 μM (+)-butaclamol is used to define specific binding, rather than to a single D_2 receptor site as previously thought [1]; we find that the baseline of 3 μM (±)-sulpiride permits the detection of a single population of sites.

This study on [^{3}H]haloperidol was instigated by recent observations that [^{3}H]spiperone is not as selective for D_2 dopamine receptors as originally thought [3] but also labels serotonin receptors, adrenoceptors and spirodecanone sites [4–6].