Local proCPU (TAFI) Activation during Thrombolytic Treatment in a Dog Model of Coronary Artery Thrombosis can be Inhibited with a Direct, Small Molecule Thrombin Inhibitor (Melagatran)

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TAFI, thrombolysis, thrombin inhibitor, melagatran, CPU assay

Introduction
Carboxypeptidase U (CPU, EC 3.4.17.20) is a basic carboxypeptidase, which circulates in plasma as the zymogen proCPU (1, 2). This zymogen is also known as thrombin activatable fibrinolysis inhibitor (TAFI) or plasma pro-carboxypeptidase B (3). Upon activation by thrombin, the thrombin/thrombomodulin complex or plasmin, CPU is able to prolong the initial phase of fibrinolysis by cleaving C-terminal lysine residues exposed on the thrombus (fibrin) surface, thereby reducing the number of plasminogen binding sites and subsequently the level of plasmin production (4).

Clot lysis experiments in vitro have demonstrated that lysis time increases with increasing proCPU concentrations (3) and that inhibitors of CPU (5, 6) or inhibition of proCPU activation (5) facilitate clot lysis. Redlitz et al. (7) showed in a dog model of coronary artery thrombosis that thrombus formation and subsequent lysis with tissue type plasminogen activator (t-PA) led to the appearance of a carboxypeptidase activity in plasma. The impact of a CPU inhibitor on t-PA-induced thrombolysis in vivo has also been studied by other investigators (8, 9) who found a two- to three-fold potentiation of clot lysis and a shorter time to restore blood flow when combining t-PA with a CPU inhibitor.

It has also been shown, both in porcine as well as in canine models of coronary artery thrombosis, that small, direct thrombin inhibitors are able to improve the thrombolytic efficacy of t-PA (10–12). This led us to the hypothesis that the pro-fibrinolytic effects of direct thrombin inhibitors may, at least partly, be due to an inhibition of thrombin-mediated activation of proCPU. The aim of the present study was to investigate the effects of melagatran, a reversible, direct thrombin inhibitor, on the generation of active CPU in coronary vessels using a dog model of coronary artery thrombosis.

Methods
Drugs
t-PA (Actilyse®) was purchased from Boehringer Ingelheim (Germany) and dissolved in sterile water to a concentration of 1 mg/ml. Melagatran, a reversible inhibitor of thrombin [inhibition constant (Kᵢ) 2 nmol/L] (13) with a molecular mass of 429.5 dalton, was synthesized by AstraZeneca (Södertälje, Sweden). PPACK and aprotinin were purchased from Alexis Biochemicals (Läufelfingen, Switzerland) and Sigma, (St. Louis, MO, USA), respectively.
Animals

Twenty-six beagle dogs of both sexes, between 1 and 2 years of age and weighing 12–18 kg, were included in the study. This study was approved by the ethical committee for animal research at the University of Gothenburg, Sweden, and conducted in accordance with the guidelines established in Guide for the Care and Use of Laboratory Animals.

Surgical Procedures and Instrumentation

Dogs were anesthetized with intravenous (iv) sodium methohexital, 50 mg/kg (Brietal®, Lilly, Indianapolis, IN, USA) followed by 100 mg/kg of α-Chloralose (90%, Aldrich-Chemie, Steinheim, Germany). A stable level of anesthesia was maintained throughout the experiment by a continuous iv infusion of α-Chloralose (40 mg/kg per h). After induction of anesthesia, the dogs were intubated with a cuffed endotracheal tube and ventilated with room air supplemented with 10 volume percent oxygen (AGA, Göteborg, Sweden) by means of a positive-pressure respirator (Servo Ventilator 900C, Siemens Elema, Solna, Sweden). The respiratory rate was maintained at 15 cycles per min. Arterial blood gases and pH were measured throughout the experiment and, when necessary, adjusted to physiological levels by adjusting the tidal volume and/or by an infusion of sodium bicarbonate.

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The coronary thrombosis model used in this study was first described by Blair et al. (14) and later modified by other investigators (15, 16). A fourth intercostal space thoracotomy was performed on the left side, and the heart was suspended in a pericardial cradle. A 5–8 mm segment of the left anterior descending (LAD) coronary artery was carefully isolated, and a 1 mm Transonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) was placed around the LAD artery to measure coronary artery blood flow. A thrombogenic copper coil, 1.2 mm in inner diameter and 6 mm in length, was advanced, under X-ray monitoring, into LAD via a guide wire in the right carotid artery, and placed proximal to the flow probe. The guide wire was then removed.

Blood for determination of CPU activity in plasma from venous and arterial samples was collected from the great cardiac vein and from the aorta, respectively. Blood samples were collected at baseline, immediately before and after coil insertion, before and after the start of t-PA treatment, and at 5–15 min respectively. Blood samples were collected at baseline, immediately before and after coil insertion, before and after the start of t-PA treatment, and at 5–15 min intervals throughout the period of vessel patency. Blood (900 µl) was collected into a tube containing 100 µl of 3.8% sodium citrate, 5 µM PPACK, and 100 µg/ml aprotinin in order to avoid ex vivo activation of proCPU by trace amounts of plasmin or thrombin. Immediately after blood collection the sample was placed in a pre-chilled “bed-side” centrifuge and plasma was prepared by centrifugation at 10,000 x g for 5 min at 4°C, snap-frozen and stored at −70°C until analyzed for CPU activity.

Experimental Protocol

After surgery dogs were randomly assigned to one of three groups; Group 1: dogs with a thrombogenic copper coil receiving t-PA alone (n = 10), Group 2: dogs with a thrombogenic copper coil receiving t-PA together with melagatran (n = 10), and Group 3: sham-operated dogs without copper coil receiving t-PA (n = 6). In all groups t-PA was administered at a dose of 1 mg/kg infused over 20 min. Melagatran (Group 2) was given as a bolus dose (0.3 mg/kg at the same time as the start of the t-PA infusion) immediately followed by a continuous infusion of 0.15 mg/kg per hour over 180 min, at which time point the experiment was terminated and the dogs were sacrificed with an overdose of pentobarbital sodium (Apoteksbolaget, Malmö, Sweden). The doses of t-PA and melagatran used in the present study were known to be effective from previous studies using the same model (12).

The experiment was initiated after allowing the occlusive thrombus to age for 30 min (Groups 1 and 2). Vessel patency was monitored continuously during the entire experiment. Reperfusion was defined as a return of coronary blood flow to a level > 0.002 L/min for a period of at least 2 min. A subsequent reocclusion was documented when the coronary blood flow declined below 0.002 L/min for a period of > 2 min. The following parameters were evaluated in Groups 1 and 2; 1) incidence and time to reperfusion, 2) incidence and time to reocclusion, and 3) mean coronary (LAD) flow during patency. Dogs in Group 3 were evaluated only with respect to CPU activity and basal flow as they had no copper-coil-induced thrombi.

CPU Activity

The plasma samples were thawed quickly under continuous mixing and then immediately put on ice. Plasma (50 µl) was added to a tube containing 4.5 µl (16 mM) 2-guanidinoethylmercaptosuccinic acid (GEMSA, Calbiochem, La

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA</td>
<td>t-PA + melagatran</td>
<td>Sham-operated</td>
</tr>
<tr>
<td>Period</td>
<td>venous</td>
<td>arterial</td>
</tr>
<tr>
<td>0–30 min</td>
<td>1.5±0.2</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>30–60 min</td>
<td>8.6±3.5</td>
<td>3.1±2.2</td>
</tr>
<tr>
<td>60–80 min</td>
<td>22.4±4.3</td>
<td>6.5±0.9</td>
</tr>
<tr>
<td>Patency</td>
<td>9.9±1.1</td>
<td>7.0±0.8</td>
</tr>
</tbody>
</table>

Table 1  Coronary patency status and blood flow parameters from t-PA and t-PA + melagatran groups

Table 2  CPU activity (U/L) and V-A difference (diff) in plasma from venous and arterial blood at baseline (0–30 min), during thrombus formation (30–60 min), during the t-PA infusion (60–80 min) and during coronary patency

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Jolla, CA, USA) and 4.5 µL (20 mM) HEPES, pH 7.4. Plasma (50 µL) was also added to a second tube containing 4.5 µL (16 mM) GEMSA and 4.5 µL (4 mg/mL) potato tuber carboxypeptidase inhibitor (PTCI; Sigma, St Louis, MO, USA). Both tubes were incubated at room temperature for 10 min. Carboxypeptidase activity was measured using a modified version of a procedure described previously (17), which enables the accurate measurement of low carboxypeptidase activity. Fifty µL of 30 mM hippuryl-arginine substrate (Bachem Feinchemikalien, Bubendorf, Switzerland) in HEPES, pH 8.0 was incubated with 25 µL of the sample for 120 min at 37°C. The reaction was stopped by adding 50 µL of 1 M HCl. Ten µL of an internal standard dilution (α-methylhippuric acid) and 300 µL of ethyl acetate were then added. After vortex mixing and centrifugation, 200 µL of the upper layer was extracted, dried and re-dissolved in mobile phase. Hippuric acid and α-methylhippuric acid were quantified by reversed-phase high-pressure liquid chromatography (HPLC) as described previously (18). The detection limit of CPU activity on top of plasma carboxypeptidase N activity using this method is 1.0 U/L. Finally, one unit of CPU activity was defined as the amount required to hydrolyse 1 µmol of substrate per minute at 37°C under the conditions described.

The amount of CPU generated in the coronary vessels drained by the great cardiac vein during the period of patency was calculated from the equation:

$$\text{CPU} = (\text{Cp(venous plasma)} - \text{Cp(arterial plasma)}) \times Q$$

In this equation, C_p is the activity of CPU (expressed as U/L) in plasma from venous (C_p(venous plasma)) and arterial blood (C_p(arterial plasma)), and Q is the mean blood flow (L/min) in LAD during the 30-s period during which the blood sample was collected. A positive C_p(venous plasma) - C_p(arterial plasma) (V-A) difference, i.e. a higher activity of CPU in venous blood leaving the heart than in arterial blood, to the heart, indicates that CPU has been generated within the coronary vessels. The rate of CPU generation is expressed as (U/min) and is the product of activity (U/L) and the mean flow rate (L/min) during the 30-s period when the sample was collected. Total generation of CPU during patency was calculated as the area under the CPU generation–time curve (AUC) using the trapezoidal model (19). This AUC model requires a minimum of three CPU concentrations determined from blood samples collected at different time points. Therefore, for practical reasons (blood sampling and centrifugation), the patency period had to be longer than 10 min to allow for the collection of three blood samples during patency. Total CPU generation (U) was calculated as the product of rate (U/min) and total patency time (min). Finally, all calculations were based on the assumption that the blood flow in the great cardiac vein was similar to that in LAD.

Statistics

Statistical evaluations were performed using the Astute statistical program attached to Microsoft Excel (DDU Software, University of Leeds, Leeds, UK). All results are given as mean values ± standard error of the mean (SEM) and a possible significant difference in time to lysis was tested using the equal variance t-test. Differences in CPU activity between treatment groups were analyzed using the non-parametric Wilcoxon-Mann-Whitney test. Differences in frequency of reocclusions between treatment groups were tested using the Fisher Exact Probability Test.

Results

In total, 26 dogs were randomized into one of the following groups: Group 1: t-PA alone (n = 10); two dogs in this group had a patent coronary artery for less than 10 min and, therefore, could only be evaluated with respect to flow parameters and not for generated amount of CPU during patency; Group 2: t-PA in combination with the direct thrombin inhibitor melagatran (n = 10), two dogs in this group died due to ventricular fibrillation; Group 3: sham-operated dogs without copper coil (n = 6). In Groups 1 and 2 an occlusive thrombus developed in the coronary artery of all dogs within 5 to 15 min after insertion of the thrombogenic copper coil. Negligible changes in aortic pressure and heart rate were noticed following thrombus formation.

Coronary patency status and blood flow parameters from Groups 1 and 2 are summarized in Table 1. There was no significant difference in basal blood flow between the two groups; however, dogs in the t-PA group (Group 1) had significantly higher mean flow during patency likely due to a more pronounced reactive hyperaemia in the t-PA group as a result of a longer time to reperfusion. Time to reperfusion was significantly shorter when melagatran was infused together with t-PA. Moreover, only one of eight dogs in the t-PA + melagatran group had a reoclusion. The net result of faster lysis and fewer reclosures was a significantly longer patency period in Group 2 compared with Group 1. Mean flow in sham-operated dogs was 0.015±0.006 L/min and the patency period was 180 min, as no thrombi were formed in this group.

The CPU activity in plasma from venous and arterial blood was analyzed twice at baseline (0–30 min), twice during thrombus formation (30–60 min), four times during t-PA infusion and, finally, three to eight times after reperfusion, dependent on the duration of the patency period. Data from one dog, representative of the results obtained in Group 1, are shown in Figure 1. The basal CPU activity in plasma from venous and arterial blood was less than 2 U/L, which increased continuously following thrombus formation and the subsequent t-PA infusion. After termination of the t-PA infusion, CPU activity declined, and had, by the end of the experiment returned to baseline levels in plasma from venous as well as from arterial blood.
The mean CPU activity (±SEM) in plasma from venous and arterial blood in the t-PA group (n = 8) is summarized in Table 2. The mean CPU activity from all dogs in this group followed the same pattern as shown in Figure 1, i.e., a marked increase in CPU activity as a result of the copper coil insertion and t-PA infusion. In Group 1, CPU activity was also always higher in plasma from venous compared with plasma from arterial blood, indicating local CPU generation within the coronary vessels. A positive V–A difference was most pronounced during the t-PA infusion when the mean activity in plasma from venous blood was 22.4 ± 4.3 U/L compared with 6.5 ± 0.9 U/L in plasma from arterial blood (P = 0.0006).

In order to exclude the possibility that the increased CPU activity observed during the t-PA infusion was due to a general activation caused by the surgical procedure, a group of sham-operated dogs was included as a control (Group 3). These dogs had the same mean basal CPU activity as dogs in Group 1 during the first hour of the experiment. There was also an increase in CPU activity during the t-PA infusion (Fig. 2). However, in contrast to dogs in Group 1, there was no significant difference (P = 0.31) in CPU activity between venous (5.4 ± 0.8 U/ml) and arterial (4.5 ± 0.8 U/L) blood in the sham-operated group (Table 2).

The effect of melagatran on the generation of CPU in venous and arterial blood is illustrated in Figure 3. As seen with t-PA alone, there

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Average flow during patency (L/min)</th>
<th>Rate of CPU generation (U/min)</th>
<th>Total amount of generated CPU (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA</td>
<td>0.014±0.006</td>
<td>0.052±0.013</td>
<td>1.30±0.31</td>
</tr>
<tr>
<td>t-PA+melagatran</td>
<td>0.008±0.002</td>
<td>0.005±0.003</td>
<td>0.69±0.47</td>
</tr>
<tr>
<td>Sham operated</td>
<td>0.016±0.006</td>
<td>0.003±0.009</td>
<td>not applicable</td>
</tr>
</tbody>
</table>

1) P <0.0001 vs t-PA alone. 2) NS vs melagatran+t-PA. 3) 0.05>P>0.01 vs the t-PA group.
was a marked increase in CPU activity during thrombus formation (30–60 min). However, initiation of t-PA treatment together with melagatran resulted in a rapid and permanent decrease in CPU activity, which remained slightly above baseline (3–4 U/L) until the end of the experiment. Furthermore, in contrast to dogs in the t-PA group, there was a very low V-A difference after initiation of melagatran.

The mean CPU activity in venous and arterial blood and corresponding V-A difference in the eight dogs from Group 2 from which complete data sets were obtained are summarized in Table 2. As expected, there was no significant difference in basal CPU activity between Groups 1 and 2 or in CPU activity during thrombus formation, as both groups were randomized and treated identically during the first hour of the experiment. However, due to the pronounced reduction in venous CPU activity after the bolus dose of melagatran, the V-A difference during the t-PA infusion decreased from 15.9 ± 3.9 U/L, as found in the t-PA group, to 2.6 ± 1.1 U/L in dogs receiving melagatran together with t-PA (P = 0.0022). This significant difference in favour of melagatran-treated dogs was also preserved during the patency period when the mean V-A difference was 2.9 ± 0.6 and 0.3 ± 0.2 U/L in Groups 1 and 2, respectively (P = 0.0001). Control experiments have shown that melagatran, in the concentration range used in the present study, does not directly inhibit CPU (Nerme, unpublished data, 1999).

The rate of CPU generation in the coronary circulation during patency was calculated as the product of the V-A difference in CPU activity (U/L) and the blood flow in LAD (L/min). The total amount of generated CPU during patency was then calculated as the area under the rate-versus-time curve (U/min × min = U). Venous and arterial plasma samples for determination of CPU activity and corresponding V-A difference were collected four to six times per animal in the t-PA group (n = 8) and six to eight times per animal in the t-PA + melagatran group (n = 8), dependent on the length of the patency period. In total, 31, 49 and 60 observations were made in group 1, 2 and 3, respectively. The results from these calculations are summarised in Table 3. These results demonstrate that not only the rate of CPU generation but also the total amount of generated CPU in coronary vessels was significantly higher in dogs treated with t-PA alone, although this group had a patency time that was significantly shorter than that seen in Group 2.

**Discussion**

It has been reported previously that canine blood, like human blood, contains proCPU that is activated during coagulation (20). Several in vitro studies have also demonstrated that inhibition of CPU with PTCI accelerates t-PA-induced clot lysis (5). Significant activation of proCPU has also been demonstrated in dogs with electrically induced coronary artery thrombosis and concomitant lysis with t-PA (7). The physiological relevance of this proCPU activation during thrombolytic treatment was later demonstrated by Klement et al. (9) who showed that co-administration of t-PA and PTCl significantly improved t-PA-induced thrombolysis. Several studies have also shown that direct thrombin inhibitors facilitate thrombolysis and prevent reocclusions when co-administered with t-PA (11, 12, 21). However, it has not yet been shown whether these effects of direct thrombin inhibitors are associated with an inhibition of proCPU activation. In the present study, therefore, we measured several flow parameters and, with a new assay, analyzed CPU activity in plasma before, during and after thrombolytic treatment with t-PA, with and without melagatran. Ten dogs were originally randomized into group 1 and 2, respectively, and eight dogs in each group fulfilled the inclusion criteria. The risk of thrombotic occlusion of coronary blood flow inherent in this animal model carries with it an increased risk of death due to ventricular fibrillation. The death of two animals in the present study is consistent with the historical incidence estimated at approximately 14% (11/97 animals) that have been observed in animals treated with t-PA, with and without melagatran.

In order to be able to measure low CPU activity (1–10 U/L) in plasma despite the carboxypeptidase activity constitutively present in plasma and attributed to CPN (mean CPN activity in dogs: 96 ± 22 U/L (20)) a novel method was developed. A competitive carboxypeptidase inhibitor (GEMSA) was used at a concentration of 400 μmol/L, which inhibited 90% and 33% of dog CPN and CPU activity, respectively. In order to discriminate further between CPN and CPU, carboxypeptidase activity was measured in the presence of 0.1 mg/ml of PTCl, which is a specific CPU inhibitor that inhibited 5% and 88% of dog CPN and CPU activity, respectively. The difference in carboxypeptidase activity measured in the presence of GEMSA alone and in the presence of GEMSA and PTCl is a measure of the CPU activity present. However, it should be noted that all CPU activity values were measured in the presence of GEMSA. This means that the generated CPU activity *in vivo* is higher than reported since the concentration of GEMSA used to inhibit CPN also inhibited CPU by 33%.

The CPU generation in the present dog model coincides with thrombus formation (when thrombin is generated) and an increase in thrombin generation after coronary thrombolysis with t-PA (22). The link between thrombin generation and proCPU activation is further strengthened by the observation that inhibition of thrombin by melagatran attenuates CPU generation (Figure 3). An increase in CPU activity was also seen in sham-operated dogs during the t-PA infusion (Figure 2). There was, however, no difference in CPU activity between venous and arterial blood collected from the great cardiac vein and aorta, indicating that this increase was due to peripheral CPU activation probably secondary to lysis of small thrombi in surgical wounds. The possibility that proCPU was activated by t-PA in the present study is unlikely as we (unpublished data) and other investigators (4) have demonstrated that t-PA cannot activate proCPU *in vitro*.

In contrast to sham-operated dogs, there was a positive V-A difference with respect to CPU activity in t-PA-treated dogs with a coronary thrombus. This indicates a local generation of CPU in coronary vessels, both during thrombus formation as well as during the thrombolytic period. A similar increase in CPU activity during thrombus formation was also seen in Group 2. However, a bolus dose of melagatran, followed by a continuous infusion over 3 h, resulted in a rapid and sustained normalization of the CPU activity, which correlated to a significantly shorter lysis time and longer patency period. Thus, prothrombin activation or release of active thrombin from the thrombus with an ensuing activation of proCPU may be a mechanism that stabilizes the thrombus and partly counteracts the effect of t-PA by reducing the number of exposed lysine residues. It has been shown that a CPU inhibitor not only facilitates t-PA induced clot lysis (23, 24) but also enhances spontaneous thrombolysis without exogenous t-PA (25, 26). Whether thrombin inhibitors also exhibit this property could not be addressed in the present study, as the anticoagulant effect of melagatran effectively inhibited reocclusions and, thereby, prevented the possibility of studying spontaneous lysis of a reoccluded coronary artery. An increased spontaneous thrombolysis after treatment with a direct thrombin inhibitor has, however, been demonstrated in pigs with a coronary artery thrombus (10). None of the pigs treated with a combination of t-PA and saline, heparin or aspirin, showed any sign of spontaneous thrombolysis after the first incidence of reocclusion. Combined treatment with t-PA and inogatran, another direct thrombin inhibitor, however, caused a dose-dependent increase in spontaneous lysis (20, 60 and 66 per cent at low,
medium and high doses, respectively). This indicates that a thrombus, established in the presence of a direct thrombin inhibitor but not heparin or aspirin, will become more susceptible to endogenous lysis due to a reduced activation of proCPU. The discrepancy between heparin and inogatran in this study may well be explained by the poor capacity of heparin to inhibit clot-bound thrombin (27).

**In vitro** activation of proCPU in plasma from different species has recently been studied by Schatteman et al. (20). They found a CPU activity of 327 ± 157 U/L in plasma from dogs that had been clotted with CaCl₂. In the present study we found an average concentration of 9 U/L in venous blood draining the heart muscle during thrombus formation, and in the same vein 22 U/L during the t-PA infusion. Thus, thrombus formation and thrombolytic treatment with t-PA results in an average CPU concentration that is about 3 and 7%, respectively, of that obtained after a strong **in vitro** activation of proCPU in dog plasma.

In conclusion, the addition of melagatran, a direct and reversible thrombin inhibitor, together with t-PA resulted in a marked reduction in CPU generation in conjunction with thrombolytic therapy with t-PA. The effect of the thrombin inhibitor is probably indirect, via the inhibition of thrombin-mediated activation of proCPU. To what extent this inhibition of proCPU activation is responsible for the shorter lysis time and the prolonged patency period that was observed in melagatran treated dogs can, however, not be elucidated from the present study. Other mechanisms such as a direct inhibition of thrombin, and thereby inhibition of fibrin accretion to the thrombus may give similar results.

**References**


