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Carboxypeptidase B inhibitors reduce tissue factor-induced renal microthrombi in rats

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Abstract

Procarboxypeptidase B (also known as thrombin-activatable fibrinolysis inhibitor) is a recently described plasma zymogen known to be activated by thrombin in plasma. Carboxy-terminal lysine residues from partially degraded fibrin are important for the binding and activation of plasminogen, and carboxypeptidase B, an active form of procarboxypeptidase B, has been shown to inhibit fibrinolysis by eliminating these residues. The present paper investigates the effects of carboxypeptidase B inhibitors, DL-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGPA) and potato-derived carboxypeptidase inhibitor (CPI), on tissue factor (TF)-induced microthrombosis in rats. Intravenous injection of MGPA (3 mg/kg and higher) or CPI (0.3 mg/kg and higher) after microthrombi formation dramatically attenuated TF-induced glomerular fibrin deposition with an increase in plasma levels of D-dimer. These results indicate that carboxypeptidase B inhibitors can enhance endogenous fibrinolysis and reduce thrombi in the TF-induced microthrombosis model after systemic administration even after thrombi formation.

Keywords: Carboxypeptidase B; Microthrombosis; Fibrinolysis; MGPA (DL-Mercaptomethyl-3-guanidinoethylthiopropanoic acid); Potato-derived carboxypeptidase inhibitor (CPI)

1. Introduction

The fibrinolytic system plays a critical role in the degradation of fibrin clots. Fibrinolysis is initiated by partial degradation of fibrin fibers by trace amounts of plasmin in plasma (Rijken and Sakharov, 2001). Carboxy-terminal (C-terminal) lysine residues in the fibrin clots derived from partial degradation of fibrin provide high-affinity binding sites for kringle domains of plasminogen and tissue-type plasminogen activator (tPA), and this facilitates the binding of these fibrinolytic factors on the surface of fibrin clots (Bouma et al., 2001). Thus, tPA effectively activates plasminogen on the surface of fibrin clots, amplifies the generation of plasmin, and ultimately leads to the efficient fibrinolysis of fibrin clots.

Human plasma procarboxypeptidase B, a 60-kDa glycoprotein identical to thrombin-activatable fibrinolysis inhibitor (TAFI), procarboxypeptidase U and procarboxypeptidase R, is biosynthesized in the liver and secreted into circulating plasma as a zymogen (Bajzar et al., 1995; Bouma et al., 2001). Procarboxypeptidase B is activated by thrombin, whereupon it is converted to carboxypeptidase B, an active zinc metalloprotease that specifically hydrolyzes the C-terminal arginine and lysine residues of proteins. It has been reported that thrombin-catalyzed procarboxypeptidase B activation is markedly enhanced by thrombomodulin, one of the high-affinity thrombin receptors expressed on the surface membrane of endothelial cells and various other cell types (Ishii, 1994; Bajzar et al., 1996b; Esmon, 2000). Another group reported that the thrombin–thrombomodulin complex is a physiological activator of procarboxypeptidase B (Mosnier et al., 2001). Thrombomodulin also functions as a cofactor in the thrombin-catalyzed activation of protein C (Esmon, 2000), and activated protein C has been shown to enhance fibrinolysis by limiting procarboxypeptidase B activation via attenuation of thrombin production (Bajzar et al., 1996a). It was further reported that procarboxypeptidase B activation was accelerated by thrombomodulin at low concentrations but attenuated by thrombomodulin at high concentrations (Nesheim et al., 1997; Hosaka et al., 1998;

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Mosnier et al., 2001). These reports suggest that fibrinolysis might be differentially regulated by thrombomodulin in different parts of the body, depending on the local thrombomodulin concentration in the vasculature (Mosnier et al., 2001).

In vitro experiments have yielded evidence suggesting that carboxypeptidase B could be involved in the regulation of fibrinolysis through removal of C-terminal lysine residues from partially degraded fibrin. Several studies have demonstrated that the addition of purified human carboxypeptidase B to human plasma prolongs the tPA-induced clot lysis time (Bajzar et al., 1995, 1996b), while still other studies have shown that the addition of anti-procarboxypeptidase B antibody, carboxypeptidase inhibitor isolated from the potato tuber (CPI) or 2-guanidinoethylmercaptosuccinic acid (GEMSA) shortens the duration of clot lysis induced by tPA (Bajzar et al., 1995, 1996a; Nesheim et al., 1997; Hosaka et al., 1998; Mosnier et al., 2001). Accordingly, it is accepted that procarboxypeptidase B is activated in the process of thrombus formation and that carboxypeptidase B then hydrolyzes the C-terminal lysine residues on the fibrin clots. Through such a process, carboxypeptidase B could act as a physiological inhibitor of fibrinolysis and a stabilizer of fibrin clots.

Several recent in vivo studies have demonstrated that the inhibition of carboxypeptidase B enhances endogenous thrombolysis as well as tPA-induced thrombolysis in the rabbit thrombosis model. Minnema et al. (1998) observed that CPI incorporated in a clot created in an isolated segment of the jugular vein potentiated endogenous thrombolysis. However, in two other studies, infusion of CPI after thrombus formation in the isolated segment of the jugular vein (Nagashima et al., 2000) and in the abdominal aorta (Klement et al., 1999) in rabbits did not enhance endogenous thrombolysis, although the tPA-induced thrombolysis was potentiated in both cases. At present, the physiological importance of carboxypeptidase B in thrombolysis remains to be clarified in various thrombotic conditions.

The most potent physiological component eliciting thrombus formation in circulating plasma is thrombin, an enzyme generated through the coagulation cascade initiated by tissue factor (TF) (Rapaport and Rao, 1995). The observation that bacterial endotoxin and inflammatory cytokines can increase TF expression in certain cells, including monocytes and endothelial cells, strongly suggests the importance of TF-induced thrombosis in septicemia and inflammation (Semeraro and Colucci, 1997; Weiss and Rashid, 1998). Thus intravenous infusion of TF can induce a thrombotic state characterized by many microthrombi in the vessels in several organs, just as observed in septicemia (Katsuura et al., 1996; Takahashi et al., 1997). The present work was undertaken to evaluate the fibrinolytic effects of two carboxypeptidase B inhibitors, DL-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGPA) (Plummer and Ryan, 1981) and CPI, on clot lysis and on TF-induced microthrombosis in rats.

2. Materials and methods

2.1. Animals and reagents

Male Wistar rats weighing 200–300 g were purchased from Charles River Japan (Yokohama, Japan). Reagents were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated. Pentobarbital was from Abbott Laboratories (North Chicago, IL, USA). MGPA was from Calbiochem-Novabiochem (La Jolla, CA, USA). CPI was purchased from Sigma (St. Louis, MO, USA) and purified by reverse-phase chromatography as previously described (Nagashima et al., 2000). Thrombin and hippuryl-arginine (Hip-Arg) were from Sigma. Thrombomodulin was from American Diagnostica (Greenwich, CT, USA). tPA (alteplase) was from Kyowa Hakko (Tokyo, Japan). Tissue factor (Innovin) and human fibrinogen standard were from Dade International (Miami, FL, USA).

2.2. In vitro assay of rat plasma carboxypeptidase B

The inhibitory activity of MGPA and CPI on carboxypeptidase-like activity was determined using Hip-Arg as a substrate. Procarboxypeptidase in rat plasma was activated as previously described (Schatteman et al., 1999). Plasma from male Wistar rats was clotted by adding $CaCl_2$ to a final concentration of 20 mM, and then this mixture was incubated for 10 min at 37 °C, placed on ice for 1 h to obtain coagulation, and centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant was removed and aprotinin was added to make a final concentration of 100 μg/ml, and then the procarboxypeptidase B in the supernatant was activated by adding thrombin (1 U/ml) and thrombomodulin (100 ng/ml). Hip-Arg hydrolytic activity was determined in a colorimetric assay as previously reported (Hosaka et al., 1998). Carboxypeptidase B activity was evaluated as the degree to which Hip-Arg hydrolytic activity was inhibited by CPI (50 μg/ml). This was based on a previous study in rats by Schatteman et al. (1999), in which CPI completely inhibited carboxypeptidase B activity but had no inhibitory effect on carboxypeptidase N activity.

2.3. In vitro clot lysis assay using rat plasma

Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Blood samples were collected from the abdominal aorta of three rats into a syringe partially filled (1:10 total volume) with 3.8% sodium citrate. Plasma was quickly obtained by centrifugation and stored at 4 °C. The clot lysis assay was performed as described previously (Hosaka et al., 1998) with a slight modification. Rat plasma (50 μl) was added to the wells of a microtiter plate containing 50 mM Tris–HCl buffer (pH 7.4) and 1500 IU/ml tPA (10 μl, final concentration 60 IU/ml) with or without carboxypeptidase B inhibitors (10 μl), the mixture was incubated at 37 °C for 10 min, and then a fibrin clot

was formed by adding 250 mM $CaCl_2$ (20 μl, final concentration 20 mM) to the solution to reach a final volume of 250 μl. The microplate was immediately shaken and turbidity was monitored at 600 nm at 37 °C for 3 h at 5-min intervals using a microplate reader (THERMO max, Molecular Devices, Sunnyvale, CA, USA). Since the turbidity did not return to the baseline level in the turbidity–time profiles in the clot lysis assay using rat plasma under the condition described above without carboxypeptidase B inhibitors, the total clot remaining in the assay was evaluated by integrating the area under the curve above the baseline from the turbidity–time profile observed over 180 min, and expressed in arbitrary units (AU).

2.4. Ex vivo clot lysis assay in rats

Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). MGPA (3, 10, 30 mg/kg) or CPI (0.3, 1, 3, 10 mg/kg) was intravenously administered. Blood samples were collected from the jugular vein into a syringe partially filled (1:10 total volume) with 3.8% sodium citrate. The samples were taken before the administration of the compound (0 min), and at 5, 15, 30, and 60 min after administration. Plasma was quickly obtained by centrifugation and stored at 4 °C. Clot lysis assay was performed as described above.

2.5. TF-induced microthrombosis model in rats

2.5.1. Experimental procedure

Rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Tissue factor (TF) in saline was continuously infused at a dose of 0.44 μg/kg (volume 5 ml/kg) via the femoral vein for 20 min (Takahashi et al., 1997). MGPA, CPI, or tPA was intravenously administered at 25 min after the beginning of the TF infusion. The animals were killed at 45 min and the kidneys were excised and fixed in 10% neutral buffered formalin. Sections of the kidneys were histologically examined after phosphotungstic acid hematoxylin (PTAH) staining for fibrin thrombi. The percentage of glomerular fibrin deposition (%GFD) was determined as follows: 100 glomeruli were examined, and the number of glomeruli with clear fibrin deposits was expressed as a percentage. To check the formation of microthrombi in the rats infused with TF, the platelet count and plasma fibrinogen concentration were measured at 0 (pre), 20, and 45 min. The concentration of D-dimer was determined in the plasma sampled at 45 min.

2.5.2. Measurement of hematological parameters

For the measurements of platelet count and fibrinogen concentration, blood samples were collected from the jugular vein into a syringe partially filled (1:10 total volume) with 3.8% sodium citrate. Platelets in the blood were counted with an automatic cell counter (Celltac α MEK-6258, Nihon Kohden, Tokyo, Japan). For the measurement of fibrinogen concentration, plasma was quickly separated from the blood sample by centrifugation and stored at −80 °C for later assay.

In the coagulation time assay for the detection of fibrinogen in plasma, 100 μl of bovine thrombin (100 U/ml) was added to diluted plasma (200 μl) preincubated at 37 °C, and the coagulation time was measured with a coagulometer (KC-10, Amelung, Germany). The fibrinogen concentration in the samples was determined from a standard curve of human fibrinogen standard (Ci-Trol level I, Dade), with a detection limit of 0.3 mg/ml. The plasma D-dimer concentration was determined in the latex agglutination test using LPIA ACE DD dimer (Dia-Iatron, Tokyo, Japan) (Aoshima et al., 1998). The detection limit for D-dimer was 0.5 μg/ml.

2.6. Statistical analysis

Statistical analysis was carried out using Dunnett's multiple comparison test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Inhibitory activity of MGPA and CPI on rat plasma carboxypeptidase B

Fig. 1 shows the inhibitory effects of the two carboxypeptidase B inhibitors, MGPA and CPI, on carboxypeptidase B activity in rat plasma. In earlier studies, MGPA was shown to inhibit the activities of carboxypeptidase B and carboxypeptidase N (Plummer and Ryan, 1981), and CPI was shown to inhibit the activities of carboxypeptidase B and carboxypeptidase A, but not carboxypeptidase N. Therefore, carboxypeptidase B-specific activity can be evaluated by using a highly specific substrate (Hip-Arg) for

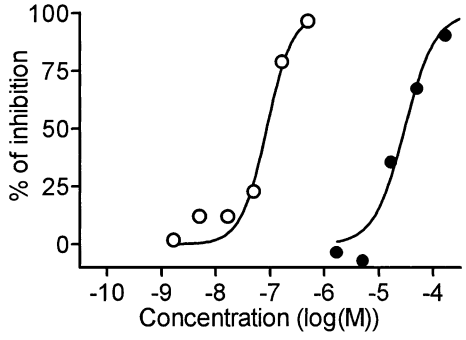

Fig. 1. Effects of MGPA and CPI on carboxypeptidase B activity in rat plasma. Rat plasma was clotted by adding $CaCl_2$ and centrifuging the mixture. Procarboxypeptidase B in the supernatant was activated by adding thrombin and thrombomodulin, and then carboxypeptidase B activity was measured as described in Materials and methods. Carboxypeptidase B activity was calculated by subtracting the Hip-Arg hydrolytic activity with CPI at a concentration of 50 μg/ml (i.e., carboxypeptidase N-like activity) from the total hydrolytic activity. Assays were performed with MGPA (●) or CPI (○).

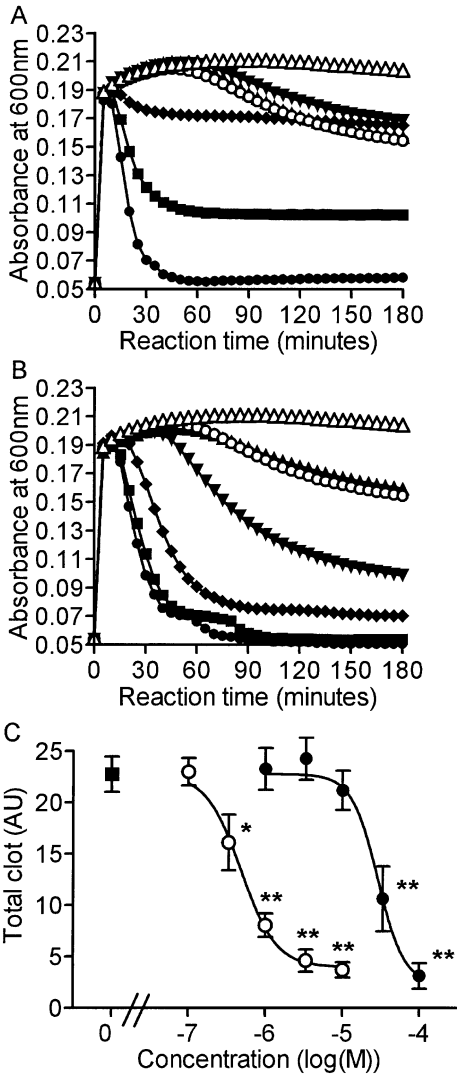

Fig. 2. Effect of MGPA or CPI on tPA-mediated clot lysis in diluted rat plasma. Clot lysis assay was performed as follows. Diluted rat plasma, 50 mM Tris–HCl buffer (pH 7.4), and tPA (final concentration 60 IU/ml) were added to the wells of a microtiter plate and fibrin clot was formed by the addition of $CaCl_2$ (final concentration 20 mM). The microplate was immediately shaken, and turbidity was monitored at 600 nm at 37 °C. (A) Effect of MGPA on clot lysis. MGPA was used at concentrations of 1 μM (▲), 3 μM (▼), 10 μM (♦), 30 μM (■), and 100 μM (●). Control assay was performed without drugs (○), and an assay without tPA was also performed (△). (B) Effect of CPI on clot lysis. CPI was used at concentrations of 100 nM (▲), 300 nM (▼), 1 μM (♦), 3 μM (■), and 10 μM (●). Control assay was performed without drugs (○), and an assay without tPA was also performed (△). Each point represents the mean of three independent tests. (C) Dose–response inhibition of MGPA (●) or CPI (○). The total clot remaining in the assay was evaluated by integrating the area under the curve above baseline from the turbidity–time profile observed over 180 min, and expressed in arbitrary units (AU). The control total clot value (■) was 22.8 ± 1.71 AU. IC_{50} values were calculated from these curves. Each point represents the mean ± S.E. of three independent experiments.

carboxypeptidase B and the inhibitor (CPI). In order to distinguish between carboxypeptidase B and carboxypeptidase N activities in plasma, procarboxypeptidase B in the plasma from nontreated rats was activated by the addition of thrombin and thrombomodulin, and carboxypeptidase B activity was determined as the degree to which the Hip-Arg hydrolysis was inhibited by excess CPI (50 μg/ml) in the present study. Both compounds, MGPA and CPI, inhibited carboxypeptidase B activity concentration dependently. IC_{50} levels of MGPA and CPI against carboxypeptidase B activity were calculated as 29.8 and 0.087 μM, respectively (Fig. 1). Higher concentrations of MGPA (>50 μg/ml) inhibited total Hip-Arg hydrolytic activity more potently than excess CPI (50 μg/ml) (data not shown). The results in rat plasma indicated, firstly, that CPI and MGPA inhibited plasma carboxypeptidase B, and secondly, that MGPA inhibited total Hip-Arg hydrolytic activity

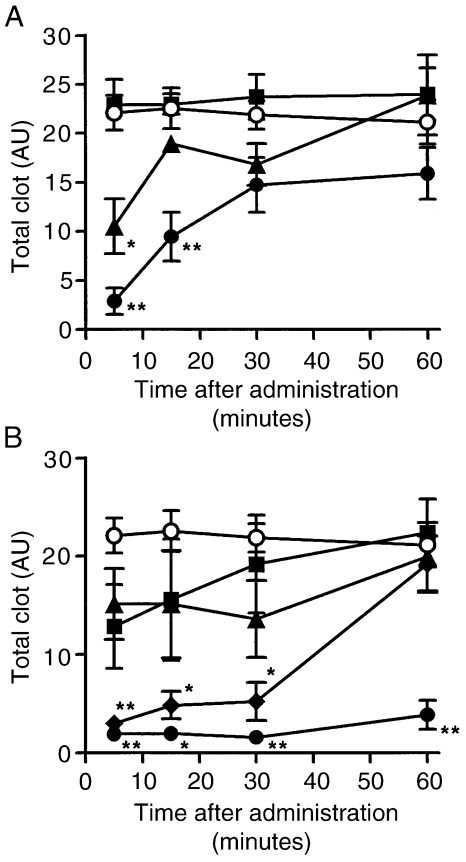

Fig. 3. Effect of MGPA or CPI on tPA-mediated ex vivo clot lysis in diluted rat plasma. Ex vivo clot lysis assay was performed as follows. Diluted rat plasma obtained by centrifugation of citrated blood samples collected from the jugular vein at various times after intravenous injection of the inhibitors, 50 mM Tris–HCl buffer (pH = 7.4), and tPA (final concentration 60 IU/ml) were added to the wells of a microtiter plate and fibrin clot was formed by the addition of $CaCl_2$ (final concentration 20 mM). The microplate was immediately shaken, and turbidity was monitored at 600 nm at 37 °C. (A) Rats were intravenously administered saline (○) or MGPA at doses of 3 mg/kg (■), 10 mg/kg (▲), and 30 mg/kg (●). (B) Rats were intravenously administered saline (○) or CPI at doses of 0.3 mg/kg (■), 1 mg/kg (▲), 3 mg/kg (♦), and 10 mg/kg (●). Plasma was sampled before drug administration (0 min), and at 5, 15, 30, and 60 min after the beginning of the experiments. Values represent means ± S.E., $n = 3–4$.

including carboxypeptidase N, as previously reported for human carboxypeptidase N (Plummer and Ryan, 1981).

3.2. Enhancement of in vitro tPA-induced clot lysis by carboxypeptidase B inhibitors

Clotting in rat plasma and subsequent tPA-induced clot lysis were continuously monitored by changes in turbidity at 600 nm in a microplate reader (Fig. 2A and B). The turbidity increased in the first 5–15 min after the addition of Ca^{2+}, and this increase represented clot formation. The increased turbidity was maintained in the plasma without tPA (Fig. 2A and B, open triangles). However, the increased turbidity slightly declined in the presence of exogenous tPA (Fig. 2A and B, open circles), and this phenomenon reflected clot lysis. MGPA at concentrations of 30 μM and higher and CPI at concentrations of 300 nM and higher intensified the decrease in turbidity. Both MGPA and CPI enhanced tPA-induced clot lysis in a dose-dependent manner, but they did not affect clot formation, as the time required for maximum clot formation did not differ from that in plasma without inhibitors (Fig. 2A and B, closed symbols). In this analysis, the 'total clot' in the course of the 180-min clot lysis assay was evaluated using the turbidity–time curve by integrating the area under the curve above baseline, expressed in arbitrary units (AU) (Fig. 2C). MGPA and CPI also reduced the 'total clot' in a dose-dependent manner. Fig. 2C shows the maximal effects of MGPA and CPI at the concentrations of 100 and 3 μM, respectively.

3.3. Enhancement of ex vivo clot lysis by carboxypeptidase B inhibitors

To estimate the pharmacologically effective doses of MGPA and CPI in vivo, the tPA-induced clot lysis assay

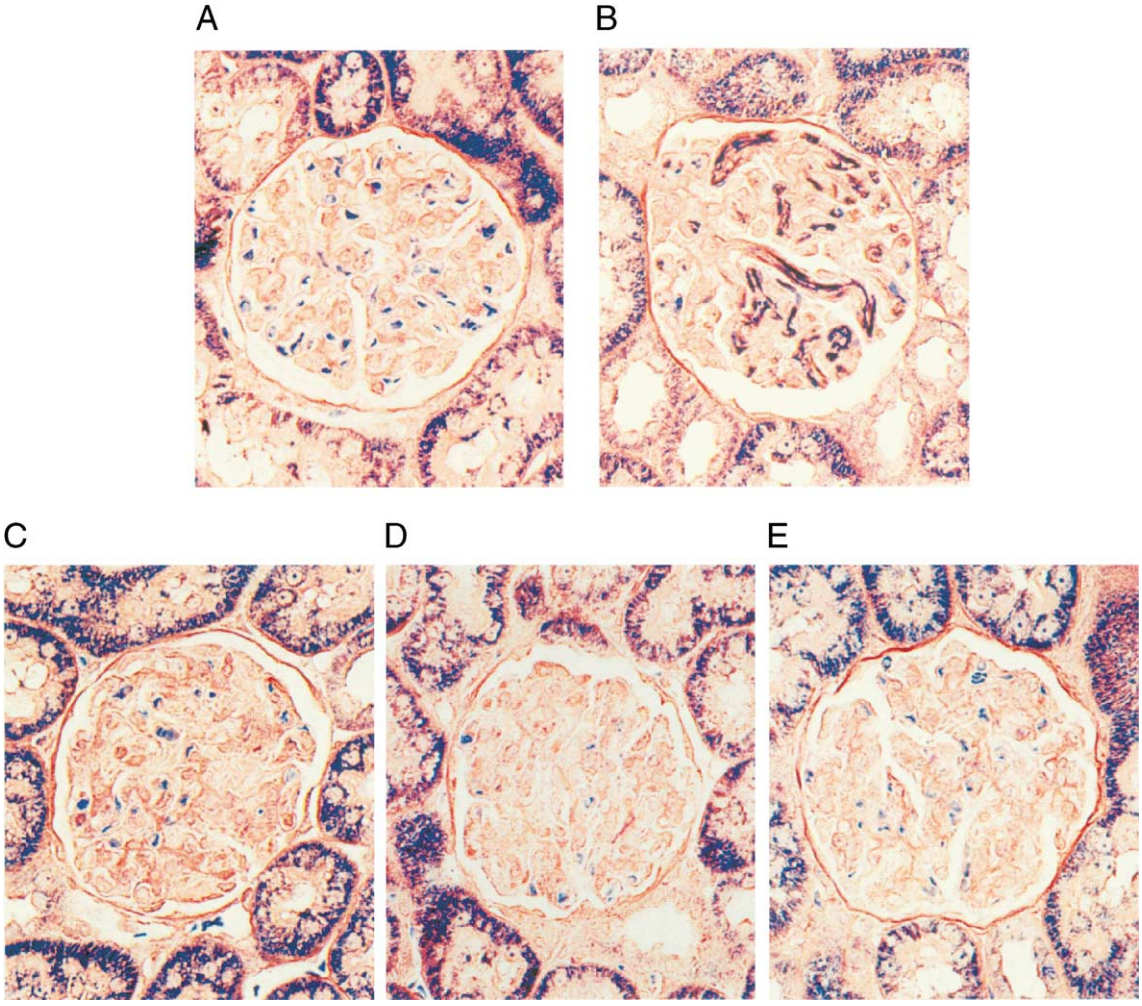

Fig. 4. Histological study of microthrombi in the kidneys of TF-treated rats. Each section was stained with PTAH for the detection of fibrin thrombi. Fibrin deposits were clearly observed in glomeruli in TF-treated (TF-control) rats (B), but not in nontreated rats (A). The fibrin deposits were clearly reduced in the rats administered MGPA at a dose of 10 mg/kg (C), CPI at a dose of 3 mg/kg (D), and tPA at a dose of 120 kIU/kg (E) 5 min after TF infusion. The original magnification was ×400.

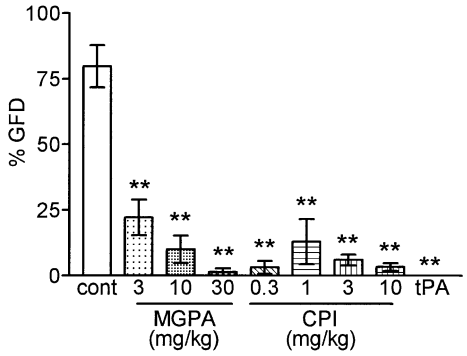

Fig. 5. Effect of MGPA, CPI, or tPA on the percentage of glomerular fibrin deposition (%GFD) in the kidneys of rats with TF-induced microthrombosis. Microthrombi were induced by sustained infusion of TF at a dose of 0.44 μg/kg for 20 min via the femoral vein. MGPA (3, 10, 30 mg/kg), CPI (0.3, 1, 3, 10 mg/kg), or tPA (120 kIU/kg) was intravenously administered at 25 min. The animals were killed at 45 min and the kidneys were excised, fixed, and stained with PTAH. The %GFD was determined by examining the number of glomeruli with fibrin deposition. Values represent means ± S.E., $n = 3–7$. $^{**}P < 0.01$ vs. TF-control (cont) was analyzed by Dunnett's multiple comparison test.

was performed using plasma from rats treated with the inhibitors (Fig. 3A and B). In the ex vivo clot lysis assay, tPA-induced clot lysis in plasma collected from rats 5 min after the administration of MGPA at doses of 10 and 30 mg/kg progressed more rapidly than that in plasma from saline-treated control rats (Fig. 3A). In the case of CPI administration as well, the enhancement of tPA-induced clot lysis was pronounced in plasma from rats administered CPI at doses of 3 and 10 mg/kg (Fig. 3B). These effects became weaker with time after the administration of the inhibitors (Fig. 3A and B). These results indicated that tPA-induced fibrinolysis in plasma was enhanced by systemic administration of MGPA and CPI in a dose- and time-dependent manner, while the plasma from control rats showed no such enhancement.

3.4. Effects of MGPA and CPI on the TF-induced microthrombosis model

In vivo effects of the carboxypeptidase B inhibitors (MGPA and CPI) were investigated in a TF-induced rat microthrombosis model by measuring fibrin deposition in the kidney (Fig. 4). In this experiment, TF was infused via the femoral vein continuously for 20 min, and the inhibitors were intravenously administered 5 min after the TF infusion. In histological studies, the rats were killed 20 min after injection of the inhibitors. PTAH staining for fibrin thrombi clearly showed more positive regions in the renal glomeruli of TF-treated rats (TF-control group) than in the glomeruli of nontreated rats (Fig. 4A and B). The percentage of glomeruli showing fibrin deposition (%GFD) in the TF-control group was 79.7%, whereas the %GFD in the nontreated group was 0% (Fig. 5). In the groups treated with MGPA (3, 10, or 30 mg/kg), CPI (0.3, 1, 3, or 10 mg/kg), or tPA (120 kIU/kg), respectively, 5 min after the TF infusion, the %GFD significantly decreased to less than 25% of that measured in the control group (Figs. 4 and 5). The antithrombotic effects of MGPA and CPI in this model (Fig. 5) were stronger than the thrombolytic effects observed in the ex vivo clot lysis experiment (Fig. 3), as the TF-induced increase in the %GFD was significantly attenuated by MGPA and CPI at doses of 3 mg/kg and higher and 0.3 mg/kg and higher, respectively (Fig. 5). These results indicated that the carboxypeptidase B inhibitors (MGPA and CPI) efficiently reduced fibrin deposition even when they were administered after the TF infusion. In order to evaluate the disseminated intravascular coagulation-like conditions in the rats during the above experiments, platelet count and fibrinogen concentration in the blood were also measured at 0 (pre), 20 (just after the end of TF infusion), and 45 min after the beginning of TF infusion (Table 1). Both parameters were clearly reduced at

Table 1
Effect of MGPA, CPI, or tPA on blood platelet count (A) and plasma fibrinogen concentration (B) in rats with TF-induced microthrombosis

(A) Platelet count ($\times 10^4/\mu l$)

Group		n	pre	20 min	45 min
TF-control		7	56.7 ± 1.85	29.8 ± 4.42	36.1 ± 1.39
MGPA	3 mg/kg	5	54.3 ± 4.03	28.0 ± 3.97	37.8 ± 3.66
	10 mg/kg	6	51.0 ± 1.88	31.3 ± 2.83	37.2 ± 3.09
	30 mg/kg	5	53.3 ± 1.56	27.1 ± 3.50	39.6 ± 2.97
CPI	0.3 mg/kg	5	55.1 ± 3.21	31.4 ± 2.46	39.6 ± 2.65
	1 mg/kg	6	50.6 ± 2.91	29.5 ± 2.43	39.8 ± 1.81
	3 mg/kg	6	61.0 ± 2.67	35.8 ± 3.37	46.1 ± 3.96
	10 mg/kg	6	55.3 ± 2.62	33.4 ± 3.07	40.2 ± 2.08
tPA	120 kIU/kg	6	53.7 ± 1.66	28.9 ± 3.86	42.0 ± 2.77
Nontreated		3	52.5 ± 3.28	54.4 ± 1.72[a]	53.8 ± 2.55[a]

(B) Fibrinogen concentration (mg/ml)

Group		n	pre	20 min	45 min
TF-control		7	1.74 ± 0.06	0.41 ± 0.04	0.33 ± 0.01
MGPA	3 mg/kg	5	1.74 ± 0.04	0.40 ± 0.10	< 0.3
	10 mg/kg	6	1.73 ± 0.04	0.47 ± 0.04	< 0.3
	30 mg/kg	5	1.71 ± 0.07	0.37 ± 0.06	< 0.3
CPI	0.3 mg/kg	5	1.81 ± 0.06	0.44 ± 0.07	< 0.3
	1 mg/kg	6	1.66 ± 0.06	0.45 ± 0.05	< 0.3
	3 mg/kg	6	1.85 ± 0.03	0.43 ± 0.04	< 0.3
	10 mg/kg	6	1.86 ± 0.03	0.46 ± 0.05	< 0.3
tPA	120 kIU/kg	6	1.77 ± 0.06	0.50 ± 0.07	< 0.3
Nontreated		3	1.75 ± 0.02	1.31 ± 0.06[b]	1.42 ± 0.05[b]

Microthrombi were induced by sustained infusion of TF at a dose of 0.44 μg/kg for 20 min via the femoral vein. MGPA (3, 10, 30 mg/kg), CPI (0.3, 1, 3, 10 mg/kg), or tPA (120 kIU/kg) was intravenously administered at 25 min. Blood was withdrawn at 0, 20, and 45 min, and the platelet count and fibrinogen concentration were determined as follows: platelets in citrated blood were counted with an automatic cell counter, and fibrinogen concentration in the plasma sample was measured with a coagulometer using clotting methods. In the measurement of fibrinogen concentration, undetectable values were calculated as 0.3 mg/ml (threshold value). Values represent means ± S.E., $n = 3–7$.

[a] $P < 0.01$ vs. TF-control, as analyzed by Student's t-test.

[b] $P < 0.05$ vs. TF-control, as analyzed by Mann–Whitney U-test.

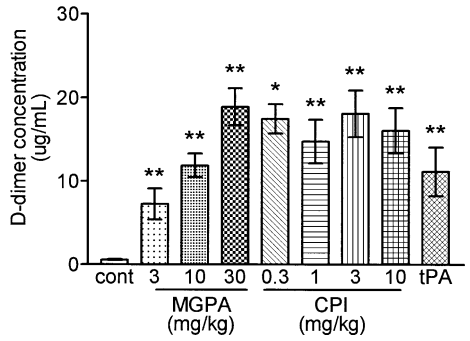

Fig. 6. Effect of MGPA, CPI, or tPA on D-dimer concentration in the plasma of rats with TF-induced microthrombosis. Microthrombi were induced by sustained infusion of TF at a dose of 0.44 μg/kg for 20 min via the femoral vein. MGPA (3, 10, 30 mg/kg), CPI (0.3, 1, 3, 10 mg/kg), or tPA (120 kIU/kg) was intravenously administered at 25 min after the beginning of TF infusion. At 45 min, blood was withdrawn from the abdominal vena cava and the plasma D-dimer concentration was determined by the latex agglutination test using LPIA ACE DD dimer. Undetectable values were calculated as 0.5 μg/ml (detection limit). Values represent means ± S.E., $n = 3–7$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. TF-control (cont) was analyzed by Steel's multiple comparison test.

20 and 45 min, and the levels of reduction were not influenced by the injection of the carboxypeptidase B inhibitors during the experimental periods, indicating that the inhibitors had no effect on the TF-induced fibrin formation or platelet decrease in the rat microthrombosis model. At the end point (45 min) of the experiment, the plasma D-dimer concentration was measured in order to clarify the fibrinolytic efficacy of the carboxypeptidase B inhibitors, as D-dimer is commonly used in clinical diagnosis as a marker of fibrinolysis. The D-dimer concentration in the TF-control group was almost the same as that in the nontreated group, i.e., almost below the detectable limit (0.5 μg/ml) (Fig. 6). The concentrations of D-dimer in the MGPA- (3, 10, or 30 mg/kg), CPI- (0.3, 1, 3, or 10 mg/kg), and tPA- (120 kIU/kg) treated groups were all significantly higher (>7.2 μg/ml) than that in the TF-treated control group, indicating that carboxypeptidase B inhibitors promoted fibrinolytic activity in the TF-induced microthrombosis model.

4. Discussion

As no specific inhibitors of carboxypeptidase B have yet been reported to be available, we used two alternatives in the present study, i.e., MGPA, a low-molecular-weight inhibitor of carboxypeptidase B and carboxypeptidase N (Plummer and Ryan, 1981), and CPI, a peptide inhibitor of carboxypeptidase B and carboxypeptidase A. MGPA and CPI were confirmed to inhibit carboxypeptidase B activity in the plasma obtained from rats (Fig. 1), and also to enhance tPA-dependent clot lysis in vitro (Fig. 2). The present ex vivo study indicated that the intravenous administration of MGPA or CPI potentiated tPA-dependent clot lysis in a dose-dependent manner (Fig. 3). In in vitro studies, the addition of carboxypeptidase B inhibitors (CPI and GEMSA) and anti-procarboxypeptidase B antibody facilitated uPA- and tPA-induced clot lysis in human plasma, while a carboxypeptidase N inhibitor (Arg-C2-Arg) and a carboxypeptidase A inhibitor (benzyl malic acid) both failed to facilitate clot lysis (Redlitz et al., 1995; Hosaka et al., 1998). This indicated that the plasminogen activator-dependent clot lysis in plasma was accelerated by carboxypeptidase B inhibitors, but not by carboxypeptidase A and carboxypeptidase N inhibitors. Therefore, the enhancement of tPA-dependent clot lysis in the plasma from MGPA- and CPI-injected rats might be attributable to the inhibition of carboxypeptidase B. The enhancement of clot lysis by systemic intravenous administration of MGPA and CPI in our ex vivo experiments using rats was consistent with the results of earlier studies performed with rabbits (Klement et al., 1999; Nagashima et al., 2000).

The present study was undertaken to evaluate the effects of carboxypeptidase B inhibitors on TF-induced microthrombosis in rats. TF infusion reduced the platelet count in blood and the fibrinogen concentration in plasma to less than 70% and 40% of the pretreatment values, respectively, indicating that microthrombi could develop in the vessels of various organs of rats (Katsuura et al., 1996; Takahashi et al., 1997). Fibrin deposition, identified as positive regions in PTAH staining (Takahashi et al., 1997; Aoshima et al., 1998), was indeed observed in the renal glomeruli of the TF-control rats (Fig. 4), and the %GFD was 79.7% versus 0% in the treated and nontreated groups, respectively (Fig. 5). In the groups treated with MGPA or CPI 5 min after TF infusion, the %GFD also markedly decreased in the rats administered tPA (Fig. 5). However, the disappearance of thrombi and the marked reduction of the %GFD resulting from the injections of MGPA, CPI, and tPA were not accompanied by any restoration of the platelet count or fibrinogen concentration that had been suppressed by the TF infusion (Table 1). Thus, these agents seemed not to inhibit prothrombotic activity, but to enhance thrombolytic activity. This apparent pattern of activity was also supported by the significant increase that we observed in the concentration of D-dimer, a clinical marker of fibrinolysis, in the plasma of TF-infused rats treated with MGPA, CPI, or tPA (Fig. 6). The D-dimer level in the control group at 45 min was below the detectable limit (0.5 μg/ml) in this experimental condition, although the level at 60 min exceeded the detectable limit (data not shown). The increase in the D-dimer level observed in the group that received MGPA was dose dependent (Fig. 6), and this was consistent with the effects on the %GFD (Fig. 5). While the effects of CPI on the %GFD and the D-dimer levels were observed from 0.3 mg/kg, the lowest dose we tested, clear dose dependent could not be shown (Figs. 5 and 6). CPI reduced the TF-induced microthrombi in a dose-dependent manner at 0.3 mg/kg and lower, whereas the maximal effect of CPI to attenuate the

increased %GFD values and to increase D-dimer levels was already observed at 0.3 mg/kg.

A number of results have been obtained in investigations of the in vivo effects of carboxypeptidase B inhibitors in thrombolysis models. One study showed the induction of lysis of a CPI-incorporated clot by reperfusion, due to enhancement of endogenous fibrinolysis, in a rabbit model of jugular vein thrombolysis (Minnema et al., 1998), whereas another study showed no lysis of a CPI-unincorporated clot created in an isolated segment of the jugular vein after systemic injection of CPI (Nagashima et al., 2000). These two reports suggest that large thrombi created in isolated segments of the jugular vein are not lysed by systemic injection of carboxypeptidase B inhibitor alone in the short period after thrombi formation, but that the lysis of a thrombus containing a carboxypeptidase B inhibitor can be induced by promoting endogenous thrombolytic activity. In contrast, our present results indicated that microthrombi such as TF-induced microthrombi can be efficiently lysed by systemic injection of carboxypeptidase B inhibitors alone through enhancement of endogenous fibrinolytic activity even after formation of thrombi. Therefore, we conclude that carboxypeptidase B inhibitors alone can be efficacious against diseases accompanied by microthrombi even if the inhibitors are administered after thrombi formation. The importance of the presence of TF-induced thrombosis in septicemia and inflammation has been strongly suggested by the observation that bacterial endotoxin, verotoxin, and inflammatory cytokines can increase TF expression in the monocytes and endothelial cells found in blood and vasculature (Fei et al., 1993; Semeraro and Colucci, 1997; Weiss and Rashid, 1998; Ishii et al., 2000). Watanabe et al. (2001) recently reported that the activity and antigen levels of TAFI/procarboxypeptidase B are significantly depressed in patients with disseminated intravascular coagulation, possibly due to the rapid clearance of carboxypeptidase B as a result of TAFI/procarboxypeptidase B activation. It has been further reported that elevated levels of procarboxypeptidase B in human plasma are correlated with symptomatic coronary artery disease and venous thrombosis (Silveira et al., 2000; van Tilburg et al., 2000). Therefore, carboxypeptidase B activated by thrombin from procarboxypeptidase B in circulating plasma might actually be associated with thrombus formation through inhibition of fibrinolysis, and carboxypeptidase B inhibitors may offer potential benefits as novel antithrombotic agents, especially against the microthrombi-related thrombosis occurring in various diseases such as bacterial infection, inflammation, thrombotic microangiopathy, and disseminated intravascular coagulation. As organ dysfunctions are clearly related to microthrombosis, drugs with a lower hemorrhage risk are needed for these diseases.

In conclusion, carboxypeptidase B inhibitors (MGPA and CPI) reduced TF-induced microthrombi in rat kidney by enhancing endogenous thrombolysis even when the inhibitors were administered after thrombi formation.

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