Characterization of Plasmin-mediated Activation of Plasma Procarboxypeptidase B

MODULATION BY GLYCOSAMINOGLYCANS*

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Plasma carboxypeptidase B (PCB) is an exopeptidase that exerts an antifibrinolytic effect by releasing C-terminal Lys and Arg residues from partially degraded fibrin. PCB is produced in plasma via limited proteolysis of the zymogen, pro-PCB. In this report, we show that the $K_m$ (55 nM) for plasmast-malyzed activation of pro-PCB is similar to the plasma concentration of pro-PCB (50–70 nM), whereas the $K_m$ for the thrombin-thrombomodulin-catalyzed reaction is 10–40-fold higher than the pro-PCB level in plasma. Additionally, tissue-type plasminogen activator triggers activation of pro-PCB in blood plasma in a reaction that is stimulated by a neutralizing antibody versus $\alpha_2$-antiplasmin. Together, these results show that plasmast-malyzed activation of pro-PCB can occur in blood plasma. Heparin (UH) and other anionic glycosaminoglycans stimulate pro-PCB activation by plasmin but not by thrombin or thrombin-thrombomodulin. Pro-PCB is a more favorable substrate for plasmin in the presence of UH (16-fold increase in $k_{cat}/K_m$). UH also stabilizes PCB against spontaneous inactivation. The presence of UH in clots prepared with prothrombin-deficient plasma delays tissue-type plasminogen activator-triggered lysis; this effect of UH on clot lysis is blocked by a PCB inhibitor from potato tubers. These results show that UH accelerates plasmast-malyzed activation of pro-PCB in plasma and PCB, in turn, stabilizes fibrin against fibrinolysis. We propose that glycosaminoglycans in the subendothelial extracellular matrix serve to augment the levels of PCB activity thereby stabilizing blood clots at sites where there is a breach in the integrity of the vasculature.

The exopeptidase activity of plasma carboxypeptidase B (PCB)$^{1,2}$ is postulated to play a key regulatory role in the fibrinolytic cascade (1, 2). The zymogen of PCB, pro-PCB, is activated by limited proteolysis to expose carboxypeptidase activity directed at C-terminal Lys and Arg residues (3, 4). Partially degraded fibrin is decorated with C-terminal basic residues that serve to accelerate fibrinolysis by providing binding sites for tissue-type plasminogen activator (t-PA), plasminogen (Plg), and plasmin (5). The ability of PCB to release C-terminal Lys and Arg residues from partially degraded fibrin thereby exerts an antifibrinolytic effect.

Thrombin was shown to activate pro-PCB albeit with low catalytic efficiency (4, 6). More recently, Bajzar et al. (7) demonstrated that thrombin-catalyzed pro-PCB activation is stimulated 1250-fold by thrombomodulin (TM), a membrane-associated thrombin-binding protein. Steady-state kinetics revealed that TM increased the turnover rate ($k_{cat}$) of the Michaelis-Menten complex, but the Michaelis constant ($K_m$) was essentially unchanged. Trypsin and plasmin were also reported to catalyze pro-PCB activation (4).

Plasmin plays a pivotal role in clot dissolution by catalyzing the proteolytic cleavage of fibrin. Plasmin-mediated fibrinolysis is subject to numerous regulatory controls to deter the bleeding diathesis that would accompany unrestricted clot lysis. In this report, we characterize a novel regulatory pathway that acts to suppress fibrinolysis. Activation of pro-PCB by plasmin to generate PCB is a negative feedback mechanism in which plasmin activity serves to dampen its familiar profibrinolytic effect. Furthermore, glycosaminoglycans (GAGs) were shown to increase markedly the rate of plasmin-mediated pro-PCB activation and stabilize PCB activity against spontaneous inactivation. A breach in the integrity of the vasculature will serve to expose GAGs that are present in the extracellular matrix (8). Hence, the GAG-mediated effects that augment the levels of PCB activity thus inhibiting fibrinolysis could possibly stabilize the clot at sites of vascular injury.

EXPERIMENTAL PROCEDURES

Materials—Pro-PCB, plasmin, and rabbit lung TM were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). The pro-PCB concentration was derived using $A_{280}$ = 1.49 (mg/ml)$^{-1}$ and an apparent relative molecular weight of 60,000 (4, 6). Sheep anti-pro-PCB IgG was from Affinity Biologicals Inc. (Hamilton, Ontario). Recombinant t-PA (Activase) was from Genentech Inc. (South San Francisco, CA). Batroxobin, goat anti-human Plg IgG, and mouse anti-$\alpha_2$-antiplasmin (2AP) monoclonal IgG were from American Diagnostica, Inc. (Greenwich, CT). Human histidine-rich glycoprotein was purified from fresh citrated plasma as described elsewhere (9). Potato carboxypeptidase inhibitor (PCI), UH from porcine intestinal mucosa (grade II; 167 USP unit/mg), low molecular weight heparin (M, 3000), heparin sulfate (i.e. heparin monosulfate), chondroitin sulfate A, chondroitin sulfate B (i.e. dermatan sulfate), chondroitin sulfate C, dextran sulfates (M, 5000 and 8000) and keratan sulfate were purchased from Sigma. The concentration of each carbohydrate was determined by the anthrone method (10, 11). 2AP and $\alpha_2$-antiplasmin were purchased from American Diagnostica, Inc. (Greenwich, CT). Human histidine-rich glycoprotein was purified from fresh citrated plasma as described elsewhere (9). Potato carboxypeptidase inhibitor (PCI), UH from porcine intestinal mucosa (grade II; 167 USP unit/mg), low molecular weight heparin (M, 3000), heparin sulfate (i.e. heparin monosulfate), chondroitin sulfate A, chondroitin sulfate B (i.e. dermatan sulfate), chondroitin sulfate C, dextran sulfates (M, 5000 and 8000) and keratan sulfate were purchased from Sigma. The concentration of each carbohydrate was determined by the anthrone method (10, 11).
centration of PCI was determined by quantitative amino acid analysis using an M_{t} of 4,300 (10). Recombinant hirudin was prepared as described elsewhere (11). 3-(2-Furyl)acryloyl-L-Ala-L-Arg (FA-AR) was purchased from Bachem Bioscience Inc. (King of Prussia, PA). The concentration of FA-AR (dissolved in deionized water) was determined using an extinction coefficient of \( \varepsilon_{280 \text{ nm}} = 1.27 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1} \) and measured on a Shimadzu UV-2101PC spectrophotometer. An exponential fit of each citrated human plasmas were from George King Biomedical, Inc. (Overland Park, KS). Prothrombin-deficient and pooled normal at room temperature in HBSP containing 2 mM CaCl_{2}. Fifty-

### Activation of Pro-PCB—Pro-PCB (50 nM) was added to 50 mM HEPES, pH 7.4, 0.15 M NaCl, and 0.1% PEG-8000 (HBSP, HEPES-buffered saline with 0.1% PEG 8000) containing CaCl_{2} (2 mM), FA-AR (720 \mu M), and various concentrations of test agents (final volume = 250 \mu l) in PEG-20,000 precoated 96-well microtiter plates. The K_{m} value of PCB for FA-AR is 360 \mu M. Plasmin or thrombin (10 \mu M) was added to initiate pro-PCB activation. The reaction was performed at room temperature with continuous monitoring of PCB activity using a Thermomax plate reader (Molecular Devices, Menlo Park, CA). PCB activity was measured in the 10–20% gradient Tris glycine SDS gels (Novex, San Diego, CA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12 or 10% acrylamide/6 M urea gels (Pierce).

#### Western Blot Analysis of Pro-PCB Activation—Pro-PCB (50 nM) was added to HBSP containing 2 mM CaCl_{2}. Other additions (where indicated) included 10 mM thrombin \( \geq 10 \) nM TM, 10 mM plasmin, and 10–100 units/ml UH (final volume = 200 \mu l). The reactions were performed at room temperature. Twenty-\mu l aliquots were removed at increasing times and quenched with concentrated Laemmli loading buffer (40 \mu l) containing 2-mercaptoethanol. Samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12 or 10–20% gradient Tris glycine SDS gels (Novex, San Diego, CA) and electrophoretically transferred to nitrocellulose membranes (Novex). Membranes were blocked with non-fat dry milk and sequentially treated with sheep anti-pro-PCB IgG and rabbit peroxidase-conjugated anti-sheep IgG (Organon Teknika Co., West Chester, PA). Pro-PCB bands were detected with the Supersignal chemiluminescent substrate (Pierce).

#### Stability of PCB Activity—Pro-PCB (500 nM) was activated with 25 nm thrombin and 25 nm TM for 40 min at 25 °C. The thrombin activity was subsequently quenched with 200 \mu M hirudin. The sample was diluted 5-fold in HBSP buffer with and without 200 units/ml UH. At various times, PCB activity was assayed with 720 \mu M FA-AR using a Shimadzu UV-2101PC spectrophotometer. An exponential fit of each data set was used to derive a first-order rate constant (k_{on}). The half-life of inactivation (t_{1/2}) was calculated from the relationship t_{1/2} = 0.693/k_{on}.

#### N-terminal Sequencing of the PCB Subunit—Pro-PCB (2 \mu M) was activated with 500 nM plasmin or 500 nM thrombin + 120 nM TM for 1 h at room temperature in HBSP containing 2 mM CaCl_{2}. Fifty-\mu l aliquots were removed and quenched with concentrated Laemmli loading buffer containing 2-mercaptoethanol. Samples were fractionated by SDS-polyacrylamide gel electrophoresis using a 12% Tris glycine SDS gel (Novex, San Diego, CA) and electrophoretically transferred to a polyvinylidene difluoride membrane (Novex). The membrane was briefly stained with Coomassie Blue, and the 35-kDa bands were visualized by Western blot analysis. The developed blots were evaluated by densitometric scanning using an Arcus II Scanner (AGFA). Data were analyzed with Adobe Photoshop software and quantified with the NIH Image program (Macintosh version).

#### Activation of Pro-PCB in Plasma—Biotinylated PCB (Biot-PCI) formed in PEG-20,000 precoated 96-well microtiter plates. The membrane was briefly stained with Coomassie Blue, and Western blots were evaluated by densitometric scanning as described above. Samples that were generated from pro-PCB by thrombin:TM was used as the standard.

#### Effect of Heparin-like Polysaccharides on Plasmin-mediated Pro-PCB Activation—Samples (50-\mu l final volume) containing 50 nm pro-PCB, 2 mM CaCl_{2}, 5 mM plasmin, 0.693/\mu M (as deduced from the primary sequence of PCI. Pooled citrated human plasma (700 \mu l) was mixed with 200 \mu M Biot-PCI and 5 \mu M Plummer’s inhibitor in HBSP buffer were placed at room temperature for 15 min. PCB was assayed by Western blot analysis and densitometry as described above. Plasmin treatment was incubated at room temperature with 50 nM PCI. Pro-PCB (200 nM) was transferred to 100 \mu l of 400 nM Biot-PCI and 100 \mu l (1.1 slurry of beads:buffer) of S-ultralin immobilized streptavidin plus beads (Sulfo, Pierce), and rotated at room temperature for 2 h. Samples were centrifuged, and the supernatants were assayed for Plg activation by Western blot analysis using an human Plg IgG. The pellets were washed with HBS, lyophilized, resuspended in SDS sample buffer, and assayed for PCB formation by Western blot analysis using the anti-pro-PCB IgG.

### RESULTS

#### Pro-PCB Is Activated by Plasmin to Yield PCB—Treatment of 50 nm pro-PCB with 10 nm plasmin generates PCB activity as measured by hydrolysis of FA-AR (Fig. 1, plot D). In contrast, 10 nm thrombin does not appear to activate PCB (Fig. 1, plot C). PCB was measured in the presence of HC-10 (200 nm) at 37 °C using a ThermoMax plate reader. The 1-h treatment period (plot A). Thrombin-mediated activation of pro-PCB is markedly increased in the presence of 10 nm TM (plot H), as shown previously (7). TM does not augment plasmin-mediated pro-PCB activation (plot E).

Pro-PCB activation was also assessed by Western blotting analysis using anti-pro-PCB IgG (Fig. 2). Pro-PCB migrates as...
Plasmin-mediated Activation of Pro-PCB

Fig. 1. Activation of pro-PCB by thrombin or plasmin. Pro-PCB (50 nM) was treated with thrombin or plasmin in HBSP at room temperature. A, 10 nM thrombin; B, 10 nM thrombin + 100 units/ml UH; C, 10 nM thrombin + 100 units/ml LMWH; D, 10 nM plasmin; E, 10 nM plasmin + 10 nM TM; F, 10 nM plasmin + 100 units/ml UH; G, 10 nM plasmin + 100 units/ml LMWH; H, 10 nM thrombin + 10 nM TM. PCB activity is evident by the decrease in A_{340 nm} due to hydrolysis of FA-AR by in situ activation of pro-PCB.

Fig. 2. Western blot analysis of pro-PCB activation using anti-pro-PCB IgG. Pro-PCB (50 nM) was incubated at room temperature in the absence (lane 1) or presence of the following: lanes 2 and 3, 10 nM thrombin for 10 and 30 min, respectively; lanes 4 and 5, 10 nM thrombin + 10 nM TM for 10 and 30 min, respectively; lanes 6 and 7, 10 nM plasmin for 10 and 30 min, respectively; lanes 8 and 9, 10 nM plasmin + 10 units/ml UH for 10 and 30 min, respectively. Treatment of pro-PCB with plasmin for 10 and 30 min (lanes 6 and 7, respectively) generates a PCB subunit band that co-migrates with that produced by thrombin.

a single band with an $M_\text{r}$ of 60,000 (lane 1). Treatment of pro-PCB with 10 nM thrombin for 10 or 30 min does not alter the mobility of the pro-PCB band (lanes 2 and 3, respectively). However, exposure of pro-PCB to thrombin:TM for 10 and 30 min (lanes 4 and 5, respectively) results in the loss of the 60-kDa band and the concomitant appearance of a 35-kDa PCB subunit protein band. Treatment of pro-PCB with plasmin for 10 and 30 min (lanes 6 and 7, respectively) generates a PCB subunit band that co-migrates with that produced by thrombin:TM.

N-terminal sequencing of the 35-kDa PCB subunits generated by thrombin:TM or plasmin was performed to map the actual cleavage sites. The first 7 amino acids obtained for the PCB subunit formed by the action of thrombin:TM (Ala-Ser-Ala-Ser-Tyr-Tyr-Glu) correspond to the sequence previously reported (4) for the thrombin-generated subunit. The plasmin-catalyzed PCB subunit yielded the same N-terminal sequence; hence, both plasmin and thrombin:TM cleave pro-PCB at Arg^{92}, Ala^{93}.

Heparin Stimulates Plasmin-mediated Pro-PCB Activation—The presence of UH (100 units/ml) during the incubation of pro-PCB and plasmin (along with FA-AR) increases the rate of hydrolysis of the PCB substrate (Fig. 1, plot F). UH does not promote thrombin-mediated pro-PCB activation (Fig. 1, plot B). The stimulatory effect of UH on plasmin-mediated pro-PCB activation is blocked by histidine-rich glycoprotein, a heparin-binding protein, in a dose-dependent manner (data not shown). UH does not alter the activity of PCB toward FA-AR (data not shown). The insensitivity of plasmin-catalyzed small substrate (S-2390) hydrolysis to the presence of UH (data not shown) together with the previously observed binding of UH to pro-PCB (14) are consistent with the view that binding of UH to pro-PCB increases the susceptibility of pro-PCB to plasmin-catalyzed activation.

Western blot analysis provides direct evidence that UH increases the rate of plasmin-mediated processing of pro-PCB. Note the more abundant 35-kDa PCB subunit at 10 and 30 min in the presence of 10 units/ml UH (Fig. 2, lanes 8 and 9, respectively) or 100 units/ml (lanes 10 and 11, respectively) when compared with the absence of UH (lanes 6 and 7, respectively). The rates of plasmin-mediated activation of pro-PCB are similar in the presence or absence of calcium, both with and without UH (data not shown). In contrast to this calcium-independent process, pro-PCB activation by thrombin:TM requires calcium (15).

Plasmin-mediated pro-PCB activation was evaluated in the presence of increasing concentrations of UH up to 200 units/ml (Fig. 3). The stimulatory effect of UH is dose-dependent and saturable. A prominent enhancement of pro-PCB activation is observed with as little as 5 units/ml of UH (plot C). Western blot analysis confirmed that the saturation kinetics does not reflect pro-PCB depletion at the higher UH concentrations (data not shown).

UH Delays Spontaneous Inactivation of PCB Activity—Incubation of PCB at 25 °C leads to spontaneous loss of its catalytic activity (7, 16, 17). The $t_{1/2}$ for this inactivation process is 74 min (Fig. 4). The $t_{1/2}$ for the spontaneous inactivation of PCB is increased to 160 min when the presence of UH (200 units/ml) by 2.3-fold to 170 min. This result suggests that PCB binds UH as does pro-PCB.

PCB Releases Arg from Plasmin and Reduces Its Pro-PCB Processing Activity—Incubation of plasmin with catalytic amounts of PCB at 37 °C results in the release of Arg (Fig. 5). Release of 1 eq of Arg was expected since Arg is situated at the C terminus of the plasmin A subunit; however, release of greater than stoichiometric amounts of Arg is observed. Re-
lease of Arg is slightly accelerated in the presence of UH. Incubation of PCB and active-site blocked plasmin, FFR-Pln, results in a markedly different Arg release profile: the amount of released Arg reaches approximately 0.7 mol of Arg per mol of FFR-Pln. These observations show that plasmin autoproteolysis (a process that should not occur with FFR-Pln) generates additional C-terminal Arg residues that are susceptible to PCB-mediated release.

Western blot analysis shows that the rate of plasmin-catalyzed formation of the 35-kDa subunit from pro-PCB is decreased by approximately 3-fold upon pretreatment of the plasmin with PCB (compare lanes 1–5 and 6–10 in Fig. 6A, and the filled and open symbols in Fig. 6B). Our data showing that PCB inhibits the release of Arg from plasmin and compromises the ability of plasmin to process pro-PCB corrobore recent results from Nesheim and co-workers (17).

The $K_m$ Value of Plasmin for Pro-PCB Is Similar to the Plasma Pro-PCB Concentration and the $k_{cat}$ Value Is Increased by UH—Densitometric scanning of the 35-kDa PCB subunit on Western blots was used to derive the kinetic constants for the activation of pro-PCB by plasmin in the absence and presence of UH. The reactions were performed in the presence of the PCB inhibitor, Plummer’s inhibitor, to block the inhibitory effect of PCB activity on plasmin-mediated pro-PCB activation (as described above). The $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values of plasmin for pro-PCB are 55 nM, $4.4 \times 10^{-3}$ s$^{-1}$, and 0.008 $\mu$M$^{-1}$ s$^{-1}$, respectively (Table I). Interestingly, the $K_m$ value of plasmin for pro-PCB is comparable to the plasma pro-PCB concentration, 50–70 nM (1, 18). The data listed in Table I also indicate that the catalytic efficiency ($k_{cat}/K_m$) of plasmin toward pro-PCB is approximately 8-fold greater than that of thrombin alone. Moreover, UH produces a 16-fold increase in catalytic efficiency of plasmin-catalyzed processing of pro-PCB, due largely to an increase in the value of $k_{cat}$.

Other Polysaccharides Also Promote Plasmin-mediated Pro-PCB Activation—The propensity of UH to stimulate plasmin-mediated pro-PCB activation is mimicked by LMWH, the UH derivative ($M_r = 4,400$) that is prepared by controlled chemical degradation (Fig. 1, plot G). LMWH does not promote thrombin-mediated pro-PCB activation (Fig. 1, plot C). A variety of other polysaccharides were also evaluated for their effects on plasmin-mediated pro-PCB activation (Fig. 1, plot F). The final concentration of each agent was 0.5 mg/ml. All values are compared with that exhibited by UH (assigned a value of 100%). The two LMWH species (LMWH 4400 and LMWH 3000) display cofactor activities that are similar to UH. Chondroitin sulfate A (i.e. dermatan sulfate) is superior to both chondroitin sulfate A and chondroitin sulfate C. Dextran sulfate 5000 and 8000 display cofactor activities that are greater than UH. Heparan sulfate and keratan sulfate do not stimulate plasmin-mediated pro-PCB activation at 0.5 mg/ml but do so at higher concentrations (data not shown). The apparent potency differences depicted in Fig. 7 may well reflect differences in the affinities of the polysaccharides for pro-PCB.

FIG. 4. UH stabilizes PCB activity against spontaneous inactivation. Pro-PCB was activated by the thrombin-TM complex and incubated in the presence (○) or absence (●) of 200 units/ml UH at 25 °C. PCB activity was measured with FA-AR (720 μM). The curves depict a single exponential fit that was used to derive the first-order inactivation rate constants.

FIG. 5. Treatment of plasmin or active site blocked plasmin (FFR-Pln) with PCB results in Arg release. Plasmin (●), FFR-Pln (○), or plasmin in the presence of UH (●) were incubated with PCB at 37 °C. Plasmin was also incubated in the absence of PCB (○). Aliquots were removed at increasing times and assayed for Arg. The data show the molar ratio of Arg production to amount of plasmin or FFR-Pln. Samples were performed in triplicate.

FIG. 6. Removal of C-terminal Arg of plasmin decreases the rate of pro-PCB activation. A, Pro-PCB (50 nM) was treated with plasmin (5 nM) or PCB-treated plasmin (5 nM) at 25 °C. Lanes 1–5, activation of pro-PCB by plasmin for 15, 30, 60, 120, and 180 min, respectively; lanes 6–10, activation of pro-PCB by PCB-treated plasmin for 15, 30, 60, 120, and 180 min, respectively. Western blot analysis with anti-pro-PCB IgG was used to detect the generation of the 35-kDa PCB subunit. B, measurement of pro-PCB activation by plasmin (●) and PCB-treated plasmin (○) using densitometric scanning of the 35-kDa subunit shown in A. The image data was blanked against a 0-min time point not shown in A.
characterization of steady-state kinetics of pro-PCB activation

Plasmin (5–10 nM) was added to HBSP buffer containing 2 mM CaCl₂, 5 μM Plummer’s inhibitor, 0–200 units/ml UH, and various concentrations of pro-PCB for 15–30 min at room temperature. Samples were quenched with concentrated Laemmli loading buffer containing 2-mercaptoethanol, electrophoresed on 12% Tris glycine SDS gels, and transferred to nitrocellulose membranes. PCB formation was measured by immunoblotting with anti-PCB IgG and densitometric scanning the 35-kDa subunit.

| Protease | Cofactor        | Kₘ (nM) | k₉₋₀ (μM⁻¹ s⁻¹) | k₉₋₀/Kₘ (μM⁻¹ s⁻¹) |
|----------|-----------------|---------|-----------------|-------------------|
| Thrombin | None            | 2,140 ± 590 | 0.0021 ± 0.0004 | 0.00098           |
|          | Thrombo-         | 1,100 ± 90 | 1.2 ± 0.06      | 1.2               |
|          | Plasmakinin      | 550 ± 65  | 0.61 ± 0.03     | 1.1               |
| Plasmin  | None            | 55 ± 15   | 0.00044 ± 0.0003 | 0.008             |
| UH       |                 | 20 ± 4    | 0.0026 ± 0.0002 | 0.13              |

* Data are from Bajzar et al. (7).
* Data are from Boffa et al. (20).

Fig. 7. Effects of different heparin-like polysaccharides on plasmin-mediated pro-PCB activation. Pro-PCB (50 nM) was incubated with plasmin (5 nM), polysaccharides (0.5 mg/ml), 2 mM CaCl₂, and 5 μM Plummer’s inhibitor for 15 min at room temperature. The polysaccharides are indicated. Samples were assayed for PCB formation by Western blotting using anti-pro-PCB IgG and densitometric scanning. All samples are normalized to PCB generated in the presence of UH (100%).

Plasmin Activates Pro-PCB in a Plasma Milieu—PCB that is generated de novo in plasma can be detected by sequestration with Biot-PCI, capture of PCB-Biot-PCI by immobilized streptavidin, and Western blot analysis using the anti-pro-PCB IgG. The appearance of the 35-kDa band is indicative of pro-PCB activation. The addition of t-PA to Biot-PCI-spiked normal human plasma results in a progressive time-dependent accumulation of PCB (Fig. 8A, lanes 1–3). The extent of the pro-PCB activation is increased in the presence of UH (lanes 4–6). The accumulation of the 35-kDa band is markedly enhanced when the reaction is performed in the presence of a neutralizing antibody versus α₂-AP (lanes 7–9). Hence, these data reveal that de novo generated plasmin is responsible for pro-PCB activation in t-PA spiked plasma and that the reaction in plasma is accelerated by UH.

The extent of Plg activation and concomitant plasmin formation in t-PA-spiked plasma was assessed by Western blot analysis using anti-Plg IgG (Fig. 8B). Only a small fraction of Plg is converted to plasmin in t-PA-spiked plasma as judged by the formation of relatively low amounts of the plasmin-α₂AP complex (Fig. 8B, lanes 2–4). The presence of UH does not substantially influence the rate of Plg activation (lanes 5–7). This analysis reveals that relatively low amounts of de novo generated plasmin are able to activate pro-PCB in a plasma milieu. The presence of the anti-α₂AP IgG causes a marked decrease in the intensity of the Plg band. The absence of a corresponding increase in the plasmin-α₂AP complex is expected since this antibody neutralizes α₂AP. The accelerated Plg activation observed in the presence of anti-α₂AP IgG probably arises because the “longer lived” plasmin activity (due to neutralization of α₂AP) converts Glu-Plg to Lys-Plg which is more readily activated by t-PA (19).

Plasmin-mediated Activation of Pro-PCB Modulates in Vitro Clot Lysis—The predicted antifibrinolytic effect due to UH-accelerated, plasmin-mediated pro-PCB activation was explored in plasma clot lysis experiments. Plasma clot formation was triggered with batroxobin instead of thrombin because the latter is readily neutralized by antithrombin III in the presence of UH. Moreover, prothrombin-deficient plasma was used in order to assess the potential antifibrinolytic activity of UH without a confounding influence of de novo generated thrombin. The addition of batroxobin to recalcified citrated prothrombin-deficient plasma containing a t-PA spike causes an increase in turbidity that is coincident with clot formation (Fig. 9). The time to reach maximal turbidity (Tmax) is approximately 30 min. In turn, plasmin that is generated by the action of t-PA on endogenous Plg lyzes the plasma clot and decreases the turbidity. The time from maximal turbidity until 50% approach to baseline (ΔT) is approximately 30 min. The Tmax is shortened from 30 to 10 min in the presence of 25 or 100 units/ml UH. Conversely, the ΔT values in the presence of 25 and 100 units/ml UH are prolonged from 30 to 90 and 130 min, respectively.

Fig. 8. Pro-PCB activation in t-PA-spiked plasma is mediated by plasmin and stimulated by UH. A, activation of pro-PCB in normal plasma at 37 °C after the addition of t-PA (5 μM/ml). Lanes 4–6, activation of pro-PCB at 15, 30, and 60 min, respectively; lanes A, lanes 1–3, activation of pro-PCB in the presence of UH (1 mg/ml) at 15, 30, and 60 min, respectively; lanes B, activation of Plg in t-PA-spiked plasma as detected by formation of the plasmin-α₂AP complex (Plg-α₂AP). Lane 1, no t-PA; lanes 2–4, t-PA added and aliquots removed at 15, 30, and 60 min, respectively; lanes 5–7, t-PA and UH added and aliquots removed at 15, 30, and 60 min, respectively; lanes 8–10, t-PA and anti-α₂-AP IgG added and aliquots removed at 15, 30 and 60 min, respectively. Samples were analyzed by Western blot using anti-Pro-PCB IgG that detects both Plg and Pln-α₂AP.
PCB in Fig. 9). PCI in the absence of UH has a much less pronounced stimulatory effect on the rate of clot lysis. This observation may reflect enhanced susceptibility of the uncleaved fibrin I clot (formed by batroxobin) to plasmin digestion and/or markedly reduced conversion of pro-PCB to PCB in the absence of UH. The ability of PCI to suppress the UH-induced prolongation of clot lysis using prothrombin-deficient plasma shows that UH is stimulating pro-PCB activation in a thrombin-independent manner. The results described earlier indicated that de novo generated plasmin is mediating the activation of pro-PCB in t-PA-spiked plasma. Hence, our studies establish that plasmin-mediated fibrinolysis is suppressed by a negative feedback mechanism involving plasmin-catalyzed pro-PCB activation.

**DISCUSSION**

PCB suppresses fibrinolysis by releasing C-terminal basic amino acids from partially degraded fibrin thereby down-regulating plasmin production. The UH-stimulated, plasmin-catalyzed activation of pro-PCB thus exerts a negative feedback effect that can limit plasmin formation in vivo. The potential physiological importance of plasmin as a pro-PCB activator is indicated by the steady-state kinetic parameters. The apparent $K_m$ values for plasmin-catalyzed activation of pro-PCB in the absence and presence of UH are 55 and 20 nM, respectively; both of these values are similar to the plasma concentration of pro-PCB, 50–70 nM (1, 18). In contrast, the apparent $K_m$ value for thrombin-catalyzed activation of pro-PCB, 0.5–2 μM, is 10–40-fold above the plasma concentration of pro-PCB (7, 20).

Further studies are needed to determine the relative contributions to the in vivo processing of pro-PCB by thrombin, plasmin, and their various macromolecular complexes.

We postulate that the binding of UH to pro-PCB alters the conformation and/or charge of pro-PCB thereby making it a more favorable substrate for plasmin. The purported avidity of pro-PCB for UH is consistent with the use of heparin-Sepharose for pro-PCB purification (14). It is noteworthy that UH does not facilitate thrombin-mediated pro-PCB activation. Pro-PCB contains a region (Trp210–Ser221, WWKMRMRWKNR) in GAG-binding proteins (21). UH may promote plasmin-mediated activation of pro-PCB via an interaction with this domain in pro-PCB.

The antifibrinolytic effect of UH that stems from its ability to promote plasmin-mediated pro-PCB activation could in theory compromise the pharmacologic utility of UH for thrombotic disorders. The intended anticoagulant effect of UH due to its propensity to serve as a cofactor for antithrombin III would be opposed by the ability of UH to work in concert with plasmin to promote pro-PCB activation. The typical efficacious anticoagulant plasma level of UH (22) is somewhat less than the threshold value, 5 units/ml, that exerts an unambiguous effect on plasmin-mediated pro-PCB activation. Nevertheless, the potential impact of this clot-stabilizing effect of UH especially during aggressive anticoagulant therapy with UH is deserving of further investigation.

Structural features of polysaccharides that promote plasmin-mediated pro-PCB activation can be gleaned from the results shown in Fig. 7. Heparan sulfate which exhibits lower cofactor activity than heparin has fewer O- or N-linked sulfate groups. Dextran sulfate displays the greatest cofactor activity; it is highly decorated with sulfate groups. Keratan sulfate, which contains a galactose residue with a neutral 6-hydroxymethyl group, is a weaker stimulator than chondroitin sulfates A or B, which possess glucuronic acid sugar units with an anionic 6-carboxylate group. These comparisons suggest that the anionic character of the polysaccharide is an important structural determinant of the cofactor activity. The stereochemistry of the polysaccharide also appears to influence the cofactor activity. For instance, chondroitin sulfate B, which contains an iduronic acid with the 5R configuration, is a better cofactor than chondroitin sulfates A or C, which contain glucuronic acid residues with the 5S configuration.

UH is a heterogeneous mixture of heavily sulfated, long chain acidic GAGs that is isolated from a variety of different tissue sources. In particular, GAGs are synthesized by endothelial cells and deposited both in the extracellular matrix and on the abluminal cell surface. We propose that plasmin-mediated pro-PCB activation and its stimulation by GAGs plays a key role in clot stabilization at sites of vascular injury. Premature lysis of the hemostatic clot at a breach in the integrity of the vasculature must be deterred to avoid hemorrhage. Exposure of GAGs due to vascular damage may well provide a nidus for plasmin-mediated pro-PCB activation and help satisfy this need. The GAG-stimulated, plasmin-dependent mechanism for pro-PCB activation is particularly relevant at injury sites where the endothelial cell surface is denuded and TM will be absent. On the other hand, it is desirable to suppress clot extension over adjacent “uninjured” regions that contain an intact endothelial cell surface. At these sites, plasmin-mediated pro-PCB activation may be latent due to the limited GAG exposure, and the fibrin deposited in this region would have a reduced stability. GAG-mediated enhancement of plasmin-catalyzed activation of pro-PCB could thus serve to modulate the life time of fibrin clots in accordance with their proximity to the extracellular matrix thereby promoting hemostasis.

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**REFERENCES**

1. Nesheim, M. E., Wang, W., Boffa, M. B., Nagashima, M, Morser, J., and Bajzar, L. (1997) *Thromb. Haemostasis* 78, 386–391
2. Plow, E. F., Allampallam, K., and Reddix, A. (1997) *Trends Cardiovasc. Med.* 7, 71–75
3. Hendriks, D., Wang, W., Scharpe, S., Lommaert, M.-P., and van Sande, M. (1999) *Biochim. Biophys. Acta* 1034, 86–92
4. Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., and Drayna, D. (1991) *J. Biol. Chem.* 266, 21833–21838
5. Suenson, E., Lutzen, O., and Thorsen, S. (1984) *Eur. J. Biochem.* 140, 516–522
6. Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) *J. Biol. Chem.* 270, 14477–14484
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7. Bajzar, L., Morser, J., and Nesheim, M. E. (1996) J. Biol. Chem. 271, 16603–16608
8. Lindahl, U., Kusche-Gullberg, M., and Kjellen, L. (1998) J. Biol. Chem. 273, 24979–24982
9. Kluszynski, B. A., Kim, C., and Faulk, W. P. (1997) J. Biol. Chem. 272, 13541–13547
10. Haas, G. M., and Ryan, C. A. (1982) Methods Enzymol. 80, 778–791
11. Jordan, S. P., Mao, S.-S., Lewis, S. D., and Shafer, J. A. (1992) Biochemistry 31, 5374–5380
12. Plummer, T. H., and Ryan, T. J. (1981) Biochem. Biophys. Res. Commun. 98, 448–454
13. Plummer, T. H., and Kimmel, M. T. (1980) Anal. Biochem. 108, 348–353
14. Broze, G. J., Jr., and Higuchi, D. A. (1996) Blood 88, 3815–3823
15. Sakharov, D. V., Plow, E. F., and Rijken, D. C. (1997) J. Biol. Chem. 272, 14477–14482
16. Wang, W., Hendriks, D. F., and Scharpe, S. S. (1994) J. Biol. Chem. 269, 15937–15944
17. Wang, W., Boffa, M. B., Bajzar, L. B., Walker, J. B., and Nesheim, M. E. (1998) J. Biol. Chem. 273, 27176–27181
18. Bajzar, M., Nesheim, M. E., and Tracy, P. B. (1996) Blood 88, 2093–2100
19. Hoylaerts, M., Rijken, D. C., Lijnen, H. R., and Collen, D. (1982) J. Biol. Chem. 257, 2912–2919
20. Boffa, M. B., Wang, W., Bajzar, L., and Nesheim, M. E. (1998) J. Biol. Chem. 273, 2127–2135
21. Cardin, A. D., and Weintraub, H. J. (1989) Arteriosclerosis 9, 21–32
22. Hirsh, J., and Puster, V. (1994) Circulation 89, 1449–1466
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