JPET Fast Forward. Published on November 3, 2004 as DOI: 10.1124/jpet.104.075770

Blood clearance and activity of erythrocyte-coupled fibrinolytics.

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Running title: Blood clearance and activity of RBC-coupled fibrinolytics.

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No of text pages : 30

Figures : 8

References : 38

Abstract : 250 words.

Introduction : 585 words.

Discussion: 1076 words.

Section : Cardiovascular

Non standard abbreviations: PA, plasminogen activators; tPA, tissue type plasminogen activator; rPA, retavase; RBC, red blood cells; B-LC-NHS, long-chain 6-biotinylaminocaproic acid N-hydroxysuccinimide ester; PAI, plasminogen activator inhibitor ; SA, streptavidin ; EGF, extracellular growth factor ; PPP, platelet poor plasma ; BSA, bovine serum albumin ; Fg, fibrinogen ; DAF, decay accelerating factor ; AUC, area under curve.

ABSTRACT

Conjugating tissue type plasminogen activator (tPA) to red blood cells (RBC) endows it with features useful for thromboprophylaxis. However, the optimal intensity and duration of thromboprophylaxis vary among clinical settings. To assess how the intrinsic properties of a plasminogen activator (PA) affects functions of the corresponding RBC/PA conjugate, we coupled equal amounts of tPA or retavase (rPA, a variant with an extended circulation time, lower fibrin affinity and greater susceptibility to PA inhibitors). Conjugation to RBC markedly prolonged the circulation of each PA in rats and mice, without detrimental effects on carrier RBC. The initial blood clearance of RBC/tPA was faster than RBC/rPA, yet it exerted greater fibrinolytic activity, in part due to greater resistance of tPA and RBC/tPA to plasma inhibitors *vs.* rPA and RBC/rPA observed *in vitro*. Soluble and RBC-coupled tPA and rPA exerted the same amidolytic activity, yet RBC/tPA lysed fibrin clots more effectively than RBC/rPA, notwithstanding comparable fibrinolytic activity of their soluble counterparts. Conjugation to RBC suppressed rPA's ability to be activated by fibrin, whereas the fibrin activation of RBC-coupled tPA was not hindered. Therefore, the functional profile of RBC/PA is influenced by: i) pharmacokinetic features provided by carrier RBC (e.g., prolonged circulation); ii) intrinsic PA features (e.g., clearance rate, resistance to inhibitors); and, iii) changes imposed by conjugation to RBC (e.g., loss of co-factor stimulation). These factors, different from those guiding the design of soluble PA for lysis of existing clots, can be exploited in the rational design of RBC/PA tailored for specific prophylactic indications.

The use of anticoagulants and anti-platelet agents for thromboprophylaxis is widely accepted and generally salutary (Romson et al., 1980; Lynch et al., 1995; Hennan et al., 2002). However, no available anti-thrombotic agents are free of side effects and fully effective in prevention of clotting (Clagett et al., 1995; Hirsh and Hoak, 1996). Plasminogen activators (PAs, such as tPA) may have especially desirable properties in this regard, including the capacity to lyse newly developing clots and thus improve thromboprophylaxis by dissolving clots formed despite anticoagulants and platelet inhibitors (Lam, 1991; Feigen et al., 1993; Noble and McTavish, 1996; Wooster and Luzier, 1999). However, use of PA for prophylactic purposes has been hampered by their: i) rapid elimination from the blood; ii) inability to distinguish between nascent pathogenic clots and their preformed haemostatic counterparts; and iii) diffusion into tissues where they may cause neurotoxicity, aberrant vascular remodeling and other side effects (Holvoet et al., 1993; Tsirka et al., 1995; Wang et al., 1998; Wang et al., 2003; Benchenane et al., 2004).

Using red blood cells (RBC) as carriers might help to circumvent these shortcomings (Muzykantov and Murciano, 2003). In theory, RBC-bound PAs (RBC/PA) would be afforded little access to tissues and mature retracted haemostatic clots but would enjoy prolonged intravascular circulation, enabling them to incorporate into nascent clots, which they might lyse from within. We recently affirmed some of these assumptions in animal models of arterial and venous thrombosis (Murciano et al., 2003; Ouriel, 2003). Injection of RBC/tPA prior to inciting thrombus formation dissolved nascent clots, while relatively sparing pre-existing clots. In contrast, soluble tPA was

cleared from blood and did not dissolve clots formed within minutes of administration even when given at considerably higher doses (Murciano et al., 2003). This encouraging initial set of results has motivated us to systematically study the potential utility of the RBC/PA delivery strategy, including optimizing composition, determining the scope of its applicability and delineating its limitations as an approach to thromboprophylaxis.

The intensity and duration of thromboprophylaxis are likely to vary widely in human diseases, ranging from intense and acute (e.g. interruption of unstable angina or recurrent transient ischemic attacks) to subtle and chronic (e.g., lessening the risk of deep venous thrombosis and pulmonary embolism post-surgery). Therefore, it is likely that the preferred characteristics of the RBC/PA as a prophylactic agent will differ as well. However, to date nothing is known as to whether conjugating PAs to RBC alters their critically important intrinsic biological features (e.g., stimulation by fibrin or suppression by PA inhibitors, PAI) and/or endows the RBC/PA complexes with unpredictable beneficiary or deleterious features.

Considerable effort has been expended to improve the clinical profile of PAs themselves (Verstraete et al., 1985; Jackson et al., 1992; Benedict et al., 1995; Collen and Lijnen, 2004). For example, Retavase (rPA, a mutant tPA derivative lacking the finger, EGF and kringle-1 domains) is more susceptible to inhibitors and has lower affinity for fibrin than wild-type tPA. However, due to its lack of domains recognized by hepatic and other tissue receptors, rPA circulates for a longer time than tPA, which may offset these limitations (Martin et al., 1991; Bu et al., 1992; Kohnert et al., 1992; Rijken et al., 1994; Noble and McTavish, 1996; Topol et al., 2000). In this study we examined effects of

coupling to RBC on the functional profiles of tPA *vs.* rPA and found that it causes profound, and, in some cases, unanticipated favorable alterations in the pharmacokinetics and enzymatic regulation of PAs of direct relevance to drug development.

METHODS

The following reagents were used: tissue type plasminogen activator, tPA or Alteplase or Activase, (Genentech, San Francisco, CA) and Retavase or rPA or Reteplase (Centocor, Malvern, PA); human fibrinogen (Enzyme Research Laboratories, South Bend, IN); human thrombin (Sigma, St. Louis, MO) and streptavidin or SA (Calbiochem, San Diego, CA); CNBr-digested fibrinogen and chromogenic substrates Spectrozyme-tPA and Spectrozyme-PL and PAI-1 (American Diagnostica, Greenwich, CT); Iodogen and long-chain 6-biotinylaminocaproic acid N-hydroxysuccinimide ester, B-LC-NHS (Pierce, Rockford, IL). Proteins were radiolabeled with Na (^{125}I) (Perkin-Elmer, Boston, MA) using Iodogen according to the manufacturer's instructions. Free ^{125}I was removed using a Bio-Spin 6 column (Bio-Rad Laboratory, Hercules CA). RBC were isolated by centrifugation from fresh anti-coagulated human, rat or murine blood and radio-labeled with (^{51}Cr)Cl (Perkin-Elmer, Boston, MA) as previously described (Muzykantov and Murciano, 1996).

Coupling of fibrinolytics to carrier RBC. To produce RBC/tPA and RBC/rPA conjugates, PAs and washed RBC were biotinylated by B-LC-NHS as described (Muzykantov and Murciano, 1996). Briefly biotinylated RBC (b-RBC) were mixed 1:1 with naive RBC (10% Hct) to prevent aggregation and incubated with streptavidin (SA, 10^6 molecules per b-RBC) for 30 minutes at RT. Unbound SA was removed by washing the cells three times. Radiolabeled biotinylated tPA (b-tPA) or Retavase (b-rPA) were incubated with the RBC for 30 minutes at RT (10^6 molecules per b-RBC), the unbound material was removed by washing, and the cell-associated ^{125}I-labeled b-PA was

measured. The resultant b-RBC/SA/b-PA complexes (indicated as RBC/tPA or RBC/rPA) were re-suspended in PBS containing 3% BSA to final 10% hematocrit .

Kinetics of blood clearance of PA *vs.* RBC/PA and *ex vivo* blood clot lysis. ^{125}I-tPA, ^{125}I-rPA, ^{51}Cr-RBC or dual-labeled ^{51}Cr-RBC/^{125}I-PA complexes were injected intravenously in anesthetized rats and mice (2-3 µg PA/animal). At selected times, aliquots of blood (200 µl) were drawn into heparin (0.05U/ml) and the radioactivity in the plasma and cell pellets were measured.

Blood fibrinolytic activity was measured using an *ex vivo* clot lysis assay described previously (Murciano et al., 2003). Briefly, 200 µl aliquots of blood were drawn into borosilicate glass tubes without anticoagulants either 10 or 60 min post-injection of PA, RBC/PA, or saline. The blood was rapidly mixed with a 4 µl solution containing trace amounts of ^{125}I-fibrinogen (^{125}I-Fg) and incubated for 20 min at RT to form a clot, obtaining ^{125}I-fibrin (Murciano et al., 2002). Clots were covered with 200 µl of saline and incubated for 6 h (unless specified otherwise) at 37^{o}C to permit fibrinolysis, assessed by the release of dissolved ^{125}I-fibrin fragments into supernatant.

Dissolution of blood and fibrin clots by PA and RBC/PA. To model *in vitro* prophylactic lysis of clots from within by entrapped RBC/PA, indicated doses of RBC/PA (5nM) were added into blood, plasma or fibrinogen solution prior to clotting. To test fibrinolysis of whole blood clots, aliquots of human, rat or mouse blood (200 µl) drawn in the absence anticoagulants were rapidly mixed with ^{125}I-Fg and allowed to clot (20 min at RT) as described above. Plasma and fibrin clots (200 µl) were prepared by adding ^{125}I-Fg to fresh platelet poor plasma (PPP) or phosphate buffered saline (PBS)

containing 3 mg/ml fibrinogen. Samples were then clotted by adding $CaCl_2$ and thrombin (final concentrations 20 mM and 0.2 U/ml, respectively). PBS (200 µl) was layered over the clots. Unless stated otherwise, indicated doses of soluble PA (5nM) were added to this layer (on top of the clot) in order to model therapeutic dissolution of pre-existing clots. Lysis was assessed by measuring the release of radioactivity into supernatants as described above. In a separate experiment, the same dose (5 nM) of free PA or RBC/PA was pre-incubated with 0.75-fold and 1-fold molar excess of PAI-1 for 30 min at room temperature and tested for fibrin clot lysis as described above.

Enzymatic activity of free PA and RBC/PA. The amidolytic activity of PA and RBC/PA was determined using chromogenic substrates for tPA and plasmin (Gardell et al., 1989). In the first series of experiments, 0.05 µM PA or RBC/PA (also containing 0.05 µM PA) was incubated with PA substrate, Spectrozyme tPA (0.4 mM), at 25ºC for 20 min. In the second series, PA or RBC/PA was incubated with 0.25 µM human plasminogen and plasmin substrate, Spectrozyme PL (0.5 mM), at 25^0C for 20 min. In the third series, CnBr-digested fibrinogen (100µg/ml) was added to the reaction medium. The mixtures (final volume 200 µl in all settings) were incubated for 20 min at 25^0C in V-shape plates centrifuged at 1200g for 2 min to precipitate RBC and the optical density was measured at 405 nm in 100 µl aliquots of supernatants. In experiments with free PA, the enzymes and substrates were dissolved in PBS. RBC/PA were suspended in PBS containing 3% BSA.

RESULTS.

Coupling tPA and rPA to RBC. To couple PAs to RBC in a biocompatible manner, we used a streptavidin-biotin technique that does not inhibit cell surface complement regulatory proteins DAF and CD59 (Muzykantov et al., 1991; Muzykantov and Taylor, 1994; Zaltzman et al., 1995; Muzykantov and Murciano, 1996; Muzykantov et al., 1996; Murciano et al., 2003; Muzykantov and Murciano, 2003). Biotinylation of tPA and rPA with up to 40-fold molar excess of B-LC-NHS did not compromise their fibrinolytic activity; greater incorporation of biotin partially inhibited the PA activity of rPA (Fig. 1A, open diamonds). Thus, PAs biotinylated with 5-fold molar excess of B-LC-NHS (Fig. 1A, inset) were used thereafter and indicated simply as tPA and rPA.

Streptavidin was used to couple biotinylated PAs to biotinylated RBC forming b-PA/SA/b-RBC complexes (indicated hereafter as RBC/tPA and RBC/rPA). RBC coating with trace labeled biotinylated ^{125}I-PA was proportional to RBC biotinylation up to an input concentration of 10 μM B-LC-NHS; at higher concentrations, coupling of rPA was more extensive (Fig. 1B). Therefore, RBC biotinylation was performed using 10 μM B-LC-NHS in all subsequent experiments to equalize PA concentration per RBC. Mouse, rat or human RBC biotinylated at this concentration of B-LC-NHS were coated with essentially the same amount of tPA and rPA (varying within the range of 6-8x10^4 PA molecules per RBC Fig. 1C). Less than 2% of the coupled ^{125}I-tPA or ^{125}I-rPA detached from the RBC over 48 hours in autologous serum.

Blood clearance of RBC/tPA and RBC/rPA. Blood levels of ^{51}Cr-RBC/tPA were ~10-20% lower than ^{51}Cr-RBC/rPA in both rats and mice 15 min post injection

(Fig. 2), but the circulation of both RBC/PAs was stable for the remainder of the experiment (3 h). Within 1 min of injection, less than 40% of the injected soluble ^{125}I-tPA dose remained in the blood of rats and mice and less that 10% remained at 15 min (Fig. 3 A and B, closed circles). In agreement with literature, ^{125}I-rPA was cleared more slowly, but by 1 h post-injection blood levels dropped to 10% and 25% of the injected dose in rats and mice, respectively (Fig. 3A and B, closed triangles).

The kinetics of RBC/^{125}I-PA was quite different. Coupling to RBC markedly prolonged circulation time of tPA and rPA (compare open *vs.* closed symbols in Fig. 3). The enhancement of blood bioavailability of RBC/tPA was somewhat greater than RBC/rPA, based on an estimate of their respective Area Under Curve (AUC) tracings. For example, in mice, the AUC of RBC/tPA was 8-fold greater than tPA, while a 5-fold difference was seen with RBC/rPA *vs.* rPA. The greater enhancement afforded by coupling of tPA to RBC relative to rPA was due to the faster clearance of free tPA *vs.* rPA. Therefore, amount of RBC/^{125}I-rPA in the blood was actually higher than seen with RBC/^{125}I-tPA in rats and mice at any inspected time point (Fig. 3).

^{125}Iodine was found mostly in blood plasma fraction after injection of free ^{125}I-tPA or ^{125}I-rPA (Fig. 4). Note that the scale shows a relative distribution of residual ^{125}I-labeled tPA and rPA in blood fractions, while their absolute level in blood was very low (see Fig. 3). When RBC/^{125}I-tPA or RBC/^{125}I-rPA were injected, approximately 90% of the radioactivity remained cell-associated for at least 3 h (Fig.4), indicating that both RBC/tPA and RBC/rPA circulate as stable complexes.

Fibrinolytic activity of circulating RBC/tPA *vs.* RBC/rPA. To characterize the activity of circulating PAs, we measured fibrinolysis *ex vivo*. To do so, ^{125}I-labeled clots were formed from fresh blood drawn at various times after injection of free PA or RBC/PA, and the release of radioactivity was measured (Murciano et al., 2003). Clot formation was not impaired in rats or mice injected with free or RBC/PA 5-180 min earlier (data not shown). Clots formed from rat blood extracted 10 min after injection of free tPA or rPA underwent 40% and 60% lysis within 6 h incubation time, respectively (Fig. 5A); therefore, the extent of fibrinolysis correlated with relative blood levels of the respective free PAs (Fig. 3A). Clots formed from rat blood extracted 1 h post injection of tPA or rPA reverted to basal levels of spontaneous fibrinolysis (Fig. 5A), consistent with the virtual complete clearance of both PAs by that time (Fig. 3A).

In sharp contrast, lysis of clots formed from rat blood drawn either 10 min or 1 h after injection of RBC/tPA or RBC/rPA was markedly accelerated (Fig. 5A). The finding that the extent of fibrinolysis induced by both RBC/PA formulations was equivalent implies that the specific activity of RBC/tPA is greater than that of RBC/rPA, because its concentration in blood was lower at the time of the assay (Fig. 3A). The same outcome was seen in mice: although the blood level of RBC/tPA was lower than RBC/rPA (Fig. 5B, left), significantly greater stimulation of *ex vivo* fibrinolysis was observed (Fig. 5B, right).

Fibrinolytic activity of free *vs.* RBC-coupled tPA and rPA *in vitro*. A series of *in vitro* studies were then conducted to investigate the discrepancy between the blood level and fibrinolytic activity of circulating RBC/tPA and RBC/rPA. First, we studied the

effect of adding the PAs directly to clots, thereby eliminating potentially confounding differences in blood clearance, species specificity, plasma inhibitors and stimulatory cofactors. Free tPA and rPA caused more extensive lysis of fibrin clots than plasma clots, likely due to effect of plasma inhibitors (Fig. 6A). This data is consistent with findings that rPA is more susceptible to inhibition by PAI-1 than tPA (Martin et al., 1991; Lijnen et al., 1994). However, free tPA and rPA caused comparable lysis of fibrin clots (Fig. 6A, inset). RBC/tPA and RBC/rPA also dissolved fibrin clots more effectively than plasma clots (Fig. 6B and C) and the activity of RBC/rPA was inhibited by plasma to a greater extent than RBC/tPA (Fig. 6B and C, left). However, in clear contrast to their soluble counterparts, RBC/tPA dissolved fibrin clots more effectively than RBC/rPA (Fig. 6B and C, right).

Coupling to RBC alters fibrin regulation of enzymatic activity of rPA, but not tPA. To determine whether this alteration in RBC/rPA activity was due to interference with the catalytic site of the enzyme, we next analyzed enzymatic activity of PA and RBC/PA using small chromogenic substrates. Both soluble and RBC-coupled tPA and rPA expressed nearly identical amidolytic activity using a tPA substrate (Fig. 7A), indicating that coupling to RBC did not directly affect the catalytic site of either enzyme. Soluble and RBC-coupled tPA and rPA also exerted comparable conversion of plasminogen into plasmin tested using a plasmin substrate (Fig. 7B), indicating that coupling to RBC did not impose conformational or steric changes that hindered cleavage of its physiological substrate, plasminogen.

Based on this outcome, we then asked whether fibrin itself might differentially regulate the activity of RBC/tPA and RBC/rPA. Soluble fibrin fragments stimulated plasmin formation by free tPA and rPA to the same extent (note scale difference between Fig. 7B and 7C). In contrast, fibrin stimulated plasmin generation by RBC/tPA to a considerably greater extent than by RBC/rPA (Fig. 7C). Therefore, coupling of rPA to RBC partially suppresses the co-factor activity of fibrin towards this mutant tPA variant.

Coupling to RBC increases tPA resistance to PAI-1. Lastly, we tested whether coupling to RBC alters susceptibility of PA to PAI-1. To do so, soluble PA or RBC/PA was incubated with buffer or with purified PAI-1 and their capacity to lyse fibrin clots *in vitro* was measured. At a PAI-1/PA molar ratio of 0.75 (Fig. 8A), free tPA was less susceptible to inhibition than rPA (~30% *vs.* >75% inhibition), as expected. Both free tPA and rPA were inhibited by >70% when pre-incubated with equimolar PAI-1 (Fig. 8B). Coupling to RBC had minimal, but rather reducing effect on rPA resistance to PAI-1 (Fig.8A). In sharp contrast, coupling tPA to RBC significantly increased tPA resistance to PAI-1, relative to free tPA: RBC/tPA was inhibited by less than 30% by an equimolar ratio of PAI-1 (Fig. 8B).

DISCUSSION

Studies in animals and humans show that RBC are attractive carriers for intravascular drug delivery (Muzykantov et al., 1996; Magnani et al., 2002; Murciano et al., 2003; Muzykantov and Murciano, 2003). This notion, initially put forth three decades ago (Ihler et al., 1973), has gained momentum due to the growing interest in cell-based therapies, including administration of stem cells as well as cells modified *ex vivo* either genetically or chemically. Linking drugs to RBC can be expected to markedly prolong their intravascular circulation and enhance their activity within blood, while concomitantly reducing their diffusion into tissues which may be the site of dose-limiting toxicity (Muzykantov et al., 1996; Bax et al., 2000; Murciano et al., 2003).

We have exploited these concepts successfully by linking plasminogen activators such as tPA to RBC. Consistent with expectations, linking tPA to RBC markedly increased its intravascular half-life, permitting selective dissolution of nascent clots while physically restricting the penetration of the drug into pre-existing clots. This effectively changed the therapeutic profile of tPA from a means to lyse existing clots into a prophylactic agent capable of preventing thrombosis over a relatively prolonged period of time (Murciano et al., 2003).

The clinical requirements for prophylaxis vary widely depending upon the intensity and duration of the pro-thrombotic stress. For example, acute and intense local release of thrombin underlies acute coronary and cerebral occlusion after plaque fissure. On the other end of the spectrum, the risk of deep venous thrombosis and pulmonary

embolism after hip or knee surgery, for example, may be associated with a less intense burst of thrombin formation but the period of risk is far more protracted. Thus, it would not be surprising if certain RBC/PA formulations were more efficacious in one or another clinical context.

Conceivably, favorable features of plasminogen activators may differ in the context of therapeutic *vs.* prophylactic mode of administration. For example, one can expect that abolishing of diffusion into clots imposed by coupling to RBC carrier would preclude therapeutic utility of RBC/PA (Sakharov and Rijken, 1995). However, the same feature, lack of penetration of RBC/PA into existing haemostatic clots and extravascular tissues, represents a safety advantage for prophylaxis (Ouriel, 2003).

There is no information, however, as to whether or, if so, how the intrinsic features of individual fibrinolytic agents translate into prophylactic efficacy when the agent is bound to RBC or other carriers. To begin to address this issue, we compared the pharmacokinetics of RBC-bound tPA (MW 60kD) *vs.* its truncated variant Retavase (MW 40kD), which has a lower affinity for fibrin and is more susceptible to plasma inhibitors, but enjoys a longer half-life in the circulation than does tPA (Martin et al., 1991; Kohnert et al., 1992; Rijken et al., 1994).

Our data show that coupling of tPA to RBC cause a modestly (<20%) accelerated clearance of the complex within first 15 min after injection, suggesting that a relatively small fraction, possibly the most heavily sensitized subpopulation of RBC/tPA, is more susceptible to elimination, primarily in the liver (data not shown). Accelerated clearance

was not seen with RBC/rPA, consistent with deletion of tissue receptor recognition domains from the variant molecule.

Nevertheless, RBC carrying either tPA or rPA are relatively stable in the bloodstream (Fig. 2) and markedly prolong circulation of the coupled PAs (Fig. 3). After injection of ^{51}Cr-RBC/^{125}I-tPA, clearance of both labels occurred in parallel (Figs. 2 and 3), indicating that both PAs circulated almost exclusively as stable RBC/PA complexes (Fig. 4). Consistent with conclusion, both RBC/tPA and RBC/rPA retained their fibrinolytic activity in the circulation for hours, whereas activity of their injected free counterparts disappeared within minutes (Fig. 5A).

Circulating RBC/tPA mediated fibrinolysis *ex vivo* more effectively than RBC/rPA, notwithstanding its faster elimination from the circulation (Fig. 5B). This difference was not due to disruption of the catalytic site caused by biotinylation as the amidolytic activity of both RBC/tPA and RBC/rPA was preserved and comparable (Fig. 7). Nor did biotinylation and RBC conjugation disrupt the capacity of RBC/rPA to bind and convert plasminogen to plasmin (Fig. 7). Rather, the enhanced specific activity of RBC/tPA is attributable not only to its relative resistance to plasma inhibitors compared with RBC/rPA (Figs. 6 and 8) but also to the preservation of fibrin-mediated co-factor activity (Fig. 7). The mechanism underlying the loss of co-factor activity of RBC/rPA is unknown but may relate to the smaller size (40 kD) and lack of auxiliary domains of the variant molecule compared with wild-type tPA (60 kD), which make the former more susceptible to modification-induced functional changes.

One important conclusion to draw from this study is that the properties desirable in a PA intended for use as a soluble fibrinolytic agent may differ from those that are most desirable in a prophylactic agent designed for prolonged intravascular activity. Our data shown that genetic modifications that prolong the survival of a soluble PA in the circulation do not contribute meaningfully to its survival once bound to RBC. Rather, RBC carriage minimizes differences in blood levels, while exacerbating the unfavorable fibrinolytic potency profile of rPA caused possibly by the lost of fibrin co-factor activity (Fig. 7). Furthermore, coupling of tPA to RBC enhanced its resistance to PAI-1, whereas it had no or even an opposite effect on rPA (Fig. 8), which helps to explain the increased specific activity of RBC/tPA *in vivo*. The molecular basis of this unexpected outcome will require additional interrogations.

Fully preserved fibrin stimulation of RBC/tPA activity is a highly desirable feature that will help to minimize potential side effects in vasculature and further augment targeting of the nascent clots, since RBC/tPA will exert relatively limited effects until it is activated by fibrin at sites of thrombosis. The enhanced resistance of RBC/tPA to PAI-1 would be expected to further it's utility as a prophylactic modality.

In summary, the data presented in this paper indicate that the pharmacological profile of RBC/PA complexes results from an interplay of diverse factors including: i) pharmacokinetic features provided by the RBC carrier (e.g., prolonged circulation, lack of extravasation); ii) intrinsic features of an individual PA (e.g., resistance to inhibitors, rate and pathway of clearance); and, iii) the newly revealed changes imposed by coupling to RBC carrier (e.g., steric limitations of interactions with plasma partners and altered

resistance to inhibitors). Greater understanding of these factors will help to further optimize RBC-based drug delivery systems and facilitate the design of RBC/PA conjugates that can be tailored to manage differing requirements to treat a range of thrombotic conditions.

Acknowledgments

Authors thank Drs. Sergei Zaitsev, Tom Dziubla and Scott Diamond (University of Pennsylvania) for helpful discussions.

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Footnotes:

This study was supported by AHA Bugher-Stroke Award, PENN Research Foundation Award and NIH RO1 HL66442 to VRM and by HL60169 to DBC. JCM was supported in part by a fellowship from the Spanish Ministry of Education and Science and by Ramon y Cajal Foundation Award.

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Legends for Figures.

Figure 1. **Coupling of tPA or Retavase to RBC**. Panel A: Fibrinolytic activity of biotinylated tPA *vs.* rPA: intact (closed and open squares), biotinylated at 40-fold (open triangles and circles) or 80-fold (closed and open diamonds) molar excess B-LC-NHS. Fibrinolysis was tested in an *in vitro* assay using ^{125} I-fibrin clots formed in tubes by addition of Ca^{++} and thrombin to fibrinogen solution. Closed triangles show rate of spontaneous fibrinolysis. Inset shows fibrinolytic activity of b_5-tPA (closed bar) and b_5-rPA (hatched bar) biotinylated at 5-fold molar excess B-LC-NHS (non-biotinylated intact counterparts are shown as b_0) after 6 hr incubation at 37^{o}C. Panel B: Coupling of ^{125}I-labeled b_5tPA (closed circles) or ^{125}I-labeled b_5rPA (open circles) via streptavidin to murine RBC biotinylated at the indicated μM inputs of B-LC-NHS. Panel C: Binding ^{125}I-labeled b_5tPA (closed bars) and ^{125}I-labeled b_5rPA (hatched bars) to RBC from different species biotinylated at 10 μM B-LC-NHS input. Data are shown as M$\pm$SD. The small bars represent binding of b_5tPA and b_5rPA to non-biotinylated RBC. In this and in all *in vitro* experiments, the data are from three independent experiments, unless otherwise indicated.

Figure 2. **Blood clearance of RBC carrying PA in rats and mice**. Kinetics of blood clearance of ^{51}Cr-labeled RBC carrying tPA (RBC/tPA, closed circles) or rPA (RBC/rPA, open circles) after intravenous injection in rats (panel A) or mice (panel B). In this and in all *in vivo* experiments, the data shown are from 4-5 mice, unless otherwise indicated.

Figure 3. **Blood clearance of free and RBC-coupled PA in rats and mice**. Kinetics of blood clearance of ^{125}I-tPA (circles) or ^{125}I-rPA (triangles) after intravenous injection in rats (A) or mice (B). Closed and open symbols show blood levels after injection of soluble *vs.* RBC-coupled plasminogen activators, respectively.

Figure 4. **Distribution of circulating PA and RBC/PA between blood plasma and cell pellet.** Distribution of circulating ^{125}I-tPA (closed and hatched bars) or ^{125}I-rPA (gray and double-hatched bars) between plasma (filled bars) and cell pellet (hatched bars) in blood samples taken 15 min after injection in rats (Panel A) or mice (Panel B). Note that the scale shows relative (%) of residual PAs in each blood fraction, while absolute amount of soluble and RBC-coupled PAs differed (see Fig. 3).

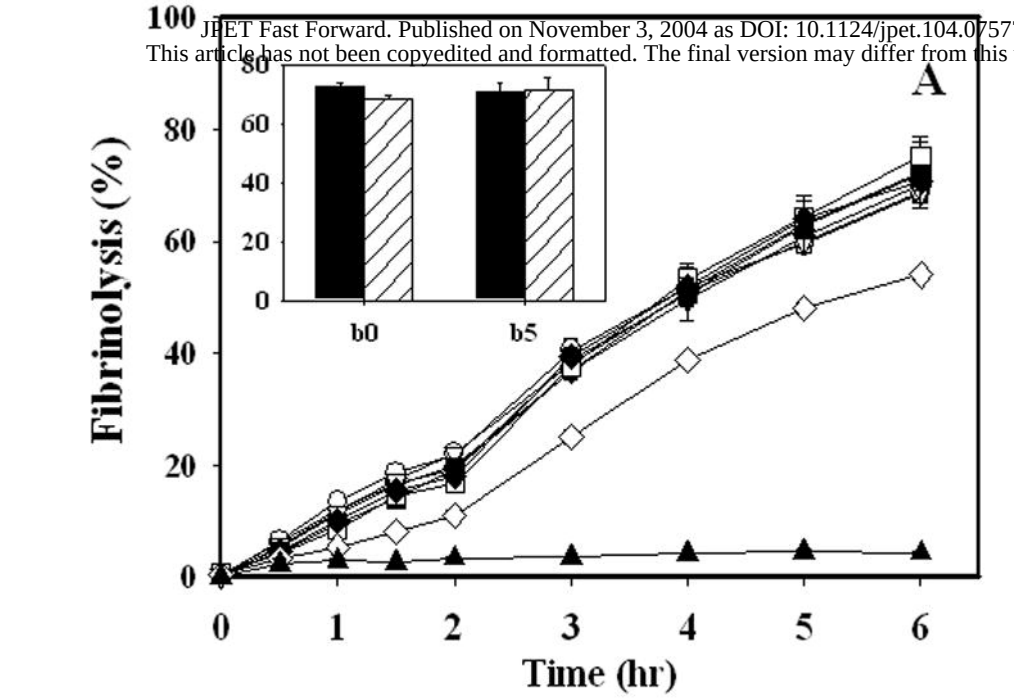
Figure 5. **Fibrinolytic activity of circulating RBC/tPA and RBC/rPA**. Panel A: *Ex vivo* dissolution of clots after 6 hrs formed from rat blood extracted either 10 or 60 min after injection of equal doses of free tPA (double-hatched bars), rPA (open bars), RBC/tPA (closed bars) or RBC/rPA (hatched bars). Panel B: Blood level of circulating RBC-coupled ^{125}I-labeled PA in mice and *ex vivo* fibrinolysis of clots after 6 hrs incubation formed from mouse blood extracted 10 min after the injection of RBC/tPA (closed) or RBC/rPA (hatched). Data shown as M$\pm$SD (n=4); *p<0.05;**p<0.01.

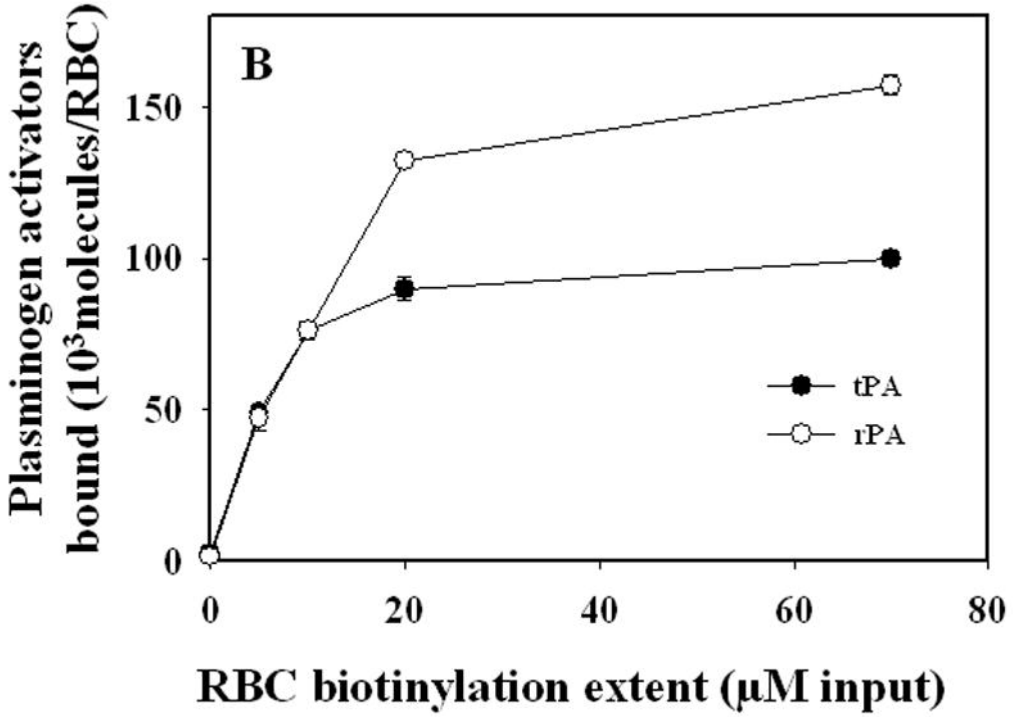
Figure 6. **Fibrinolytic activity of free PA and RBC/PA *in vitro***. Dissolution of clots formed from human plasma (left bars) or fibrin (right bars) after 6 hrs by tPA (striped bars) or rPA (closed bars). Double-hatched bars show the level of spontaneous clot lysis. Panel A: free tPA or rPA (2 nM) were added to clots. The inset shows the kinetics of fibrin clots dissolution by free tPA (open triangle) *vs.* rPA (closed circles)

after 2 hrs. Panels B and C show fibrinolysis induced by 5 nM and 2 nM RBC-coupled tPA *vs.* rPA, respectively. Data shown as M±SD (n=4); ***p*<0.01, ****p*<0.001.

Figure 7. **Enzymatic activity of free PA and RBC/PA *in vitro***. Activity of free *vs.* RBC-coupled tPA (striped bars) or rPA (closed bars) determined by proteolysis of chromogenic substrates. Double-hatched bars show the background level of spontaneous proteolysis. Panel A: Results obtained with a direct tPA substrate, Spectrozyme tPA. Panel B: Results obtained in the presence of plasminogen and the plasmin substrate, Spectrozyme Pl. Panels C: The conditions were the same as in B, but in the presence of soluble fibrin fragments. Note that free and RBC-coupled tPA and rPA display identical activity in all tests with the exception of fibrin-dependent activation of plasminogen to plasmin (Panel C, difference between RBC/tPA and RBC/rPA is significant, *****p<0.05).

Figure 8. Inhibition of fibrinolysis by free PA *vs* RBC/PA by PAI-1. Equal dose (5 nM) of either free (closed bars) or RBC-coupled (hatched bars) tPA *vs* rPA was incubated in PBS or PBS supplemented with 0.75-fold (A) or equimolar (B) amounts of PAI-1 for 30 min at RT. The lysis of fibrin clots was then measured *in vitro*. Data presented as M±SD (n=3) shows percent of fibrinolysis inhibition relative to the respective PA or RBC/PA alone. The difference in the activity of RBC/tPA and either free tPA or RBC/rPA is statistically significant, p<0.001 (***).





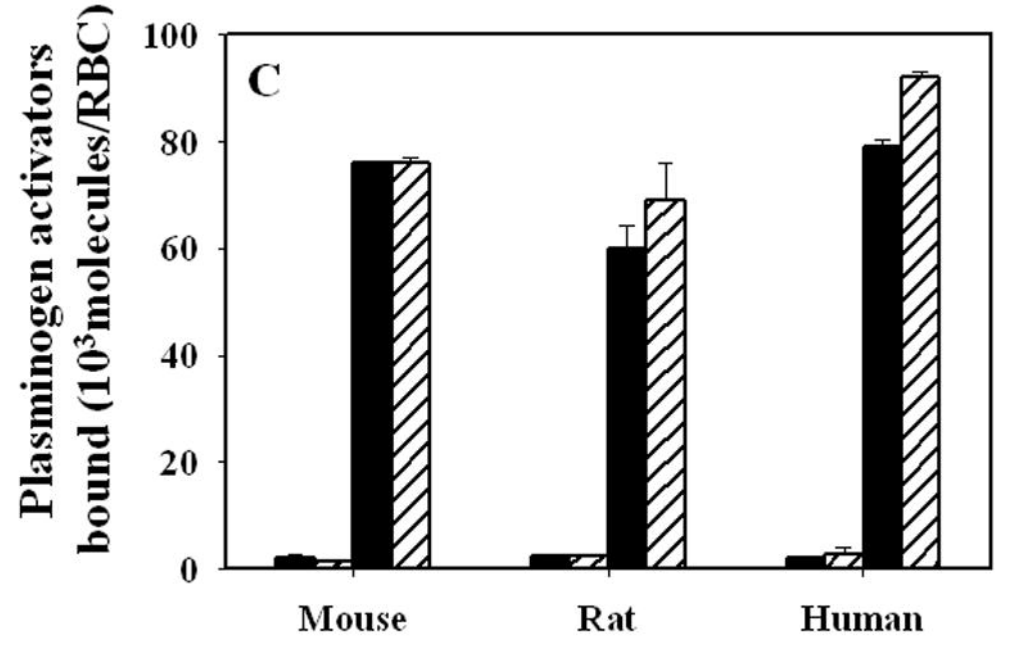


Fig 1

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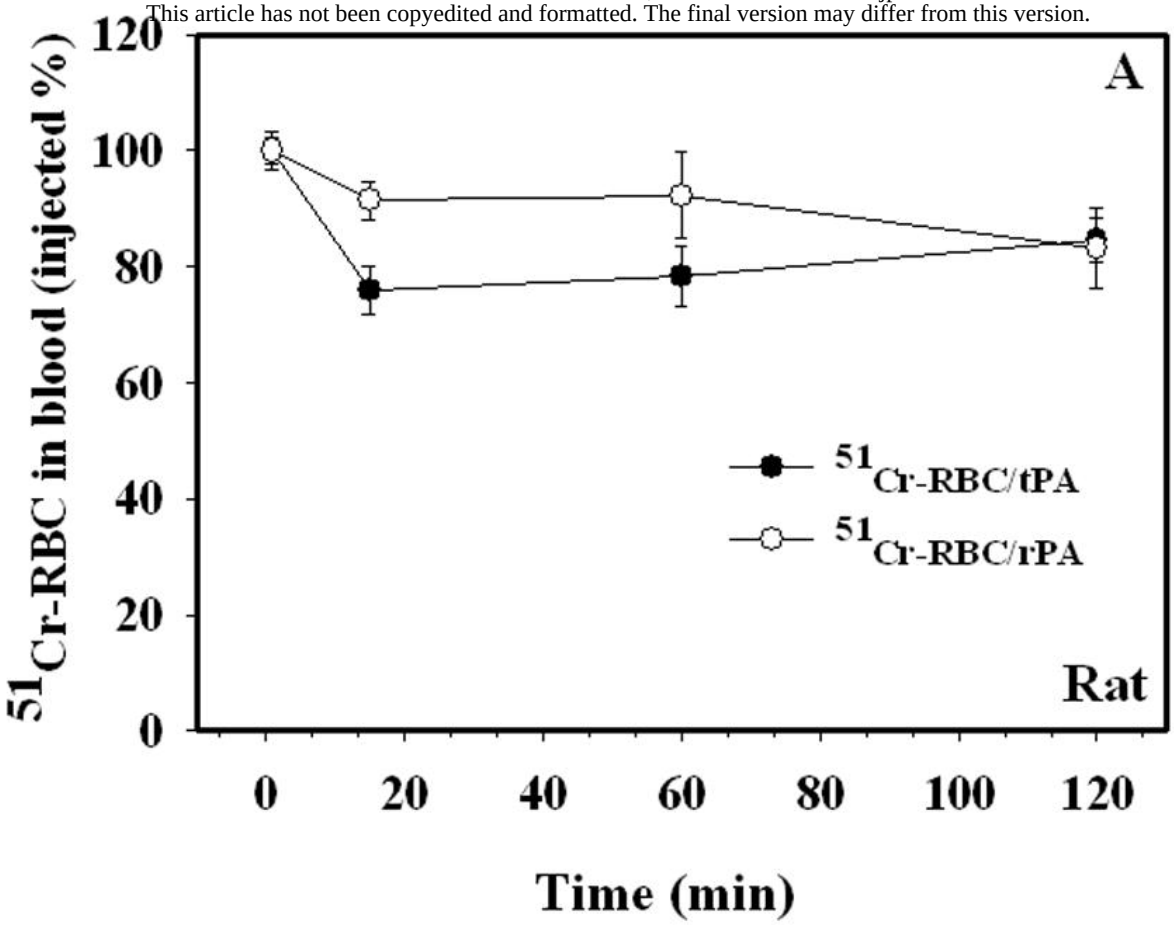

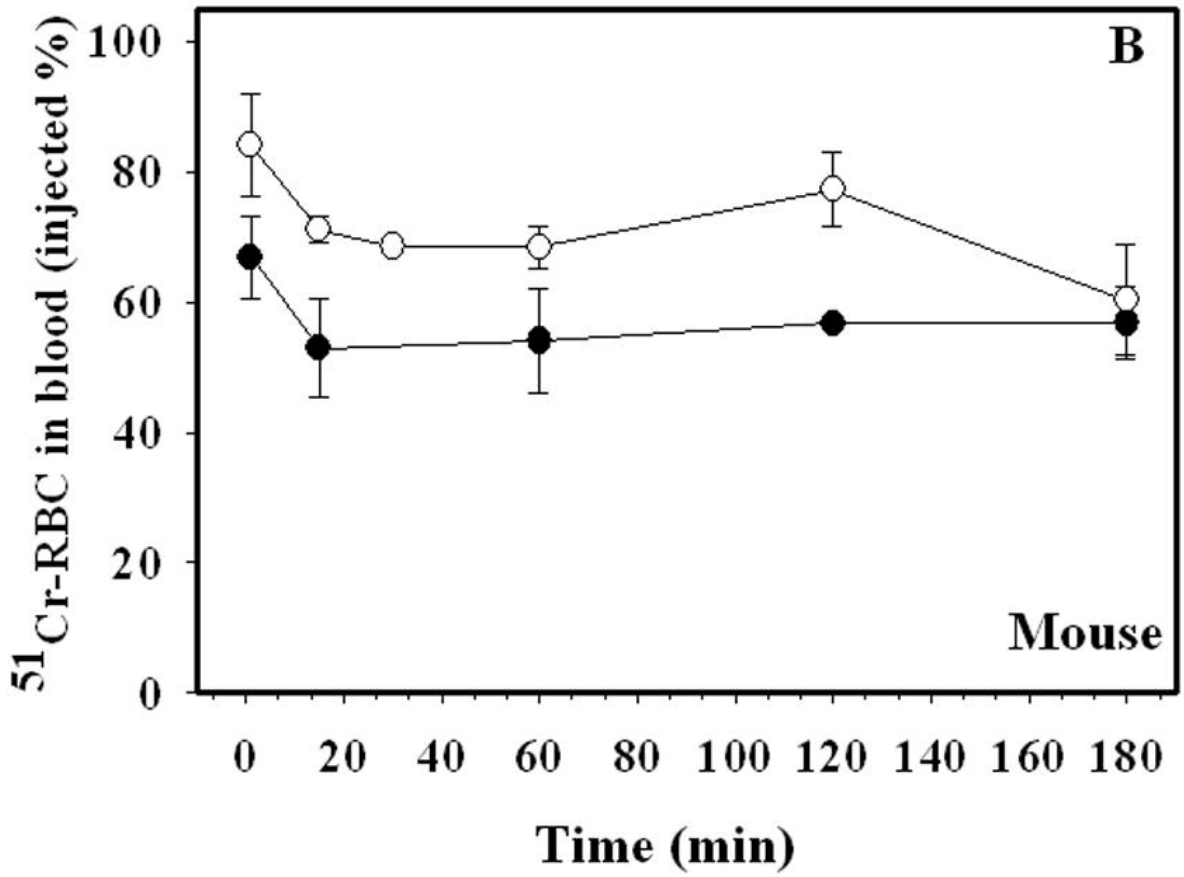

Fig 2

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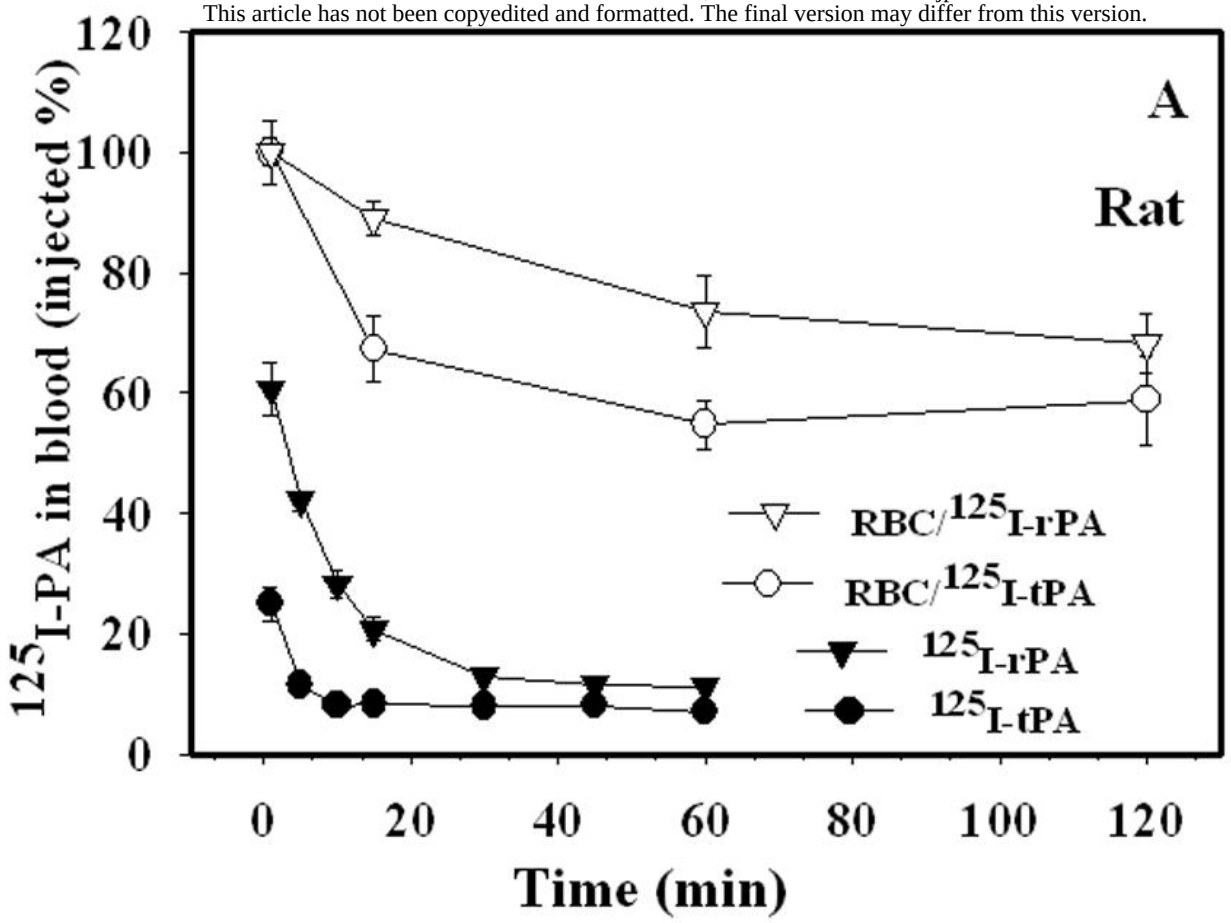

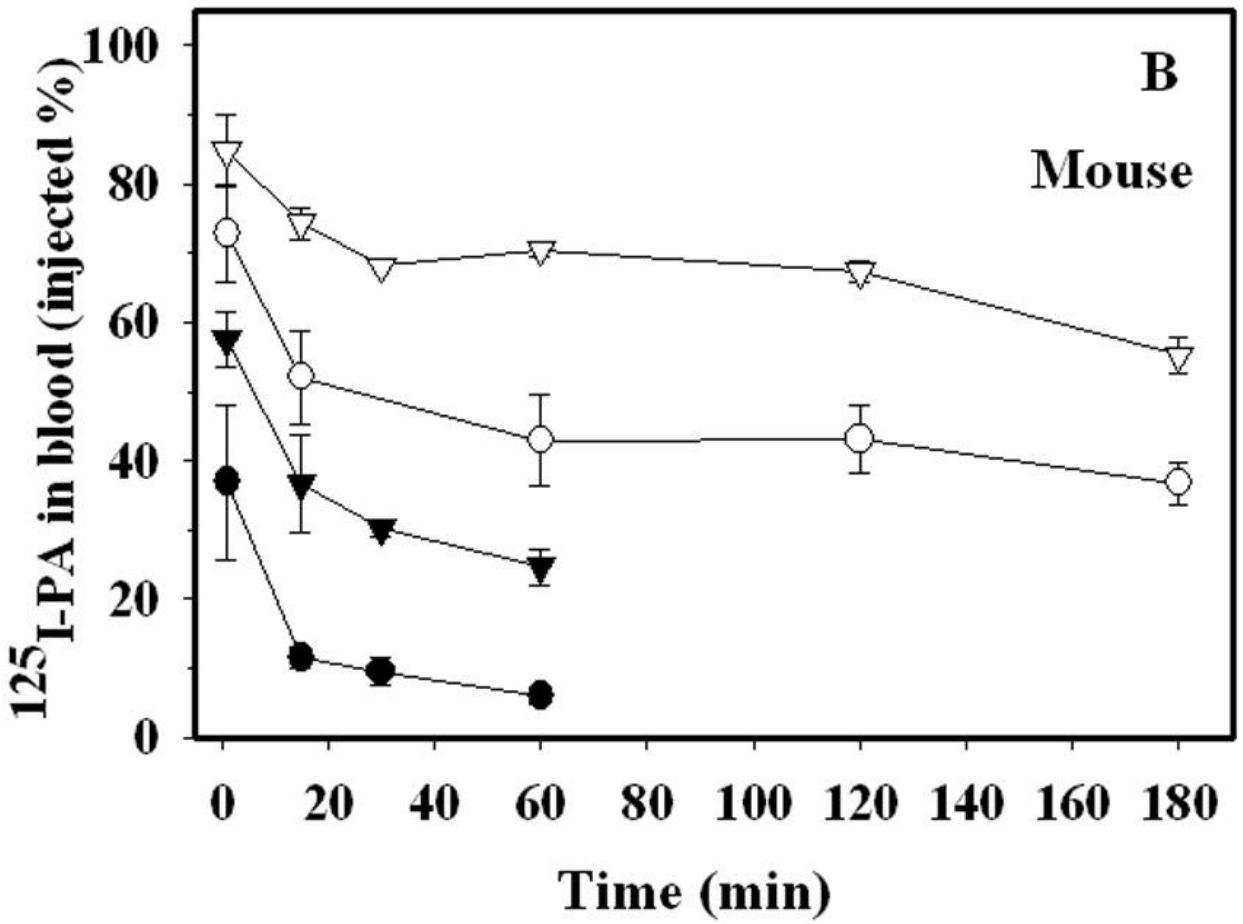

Fig 3

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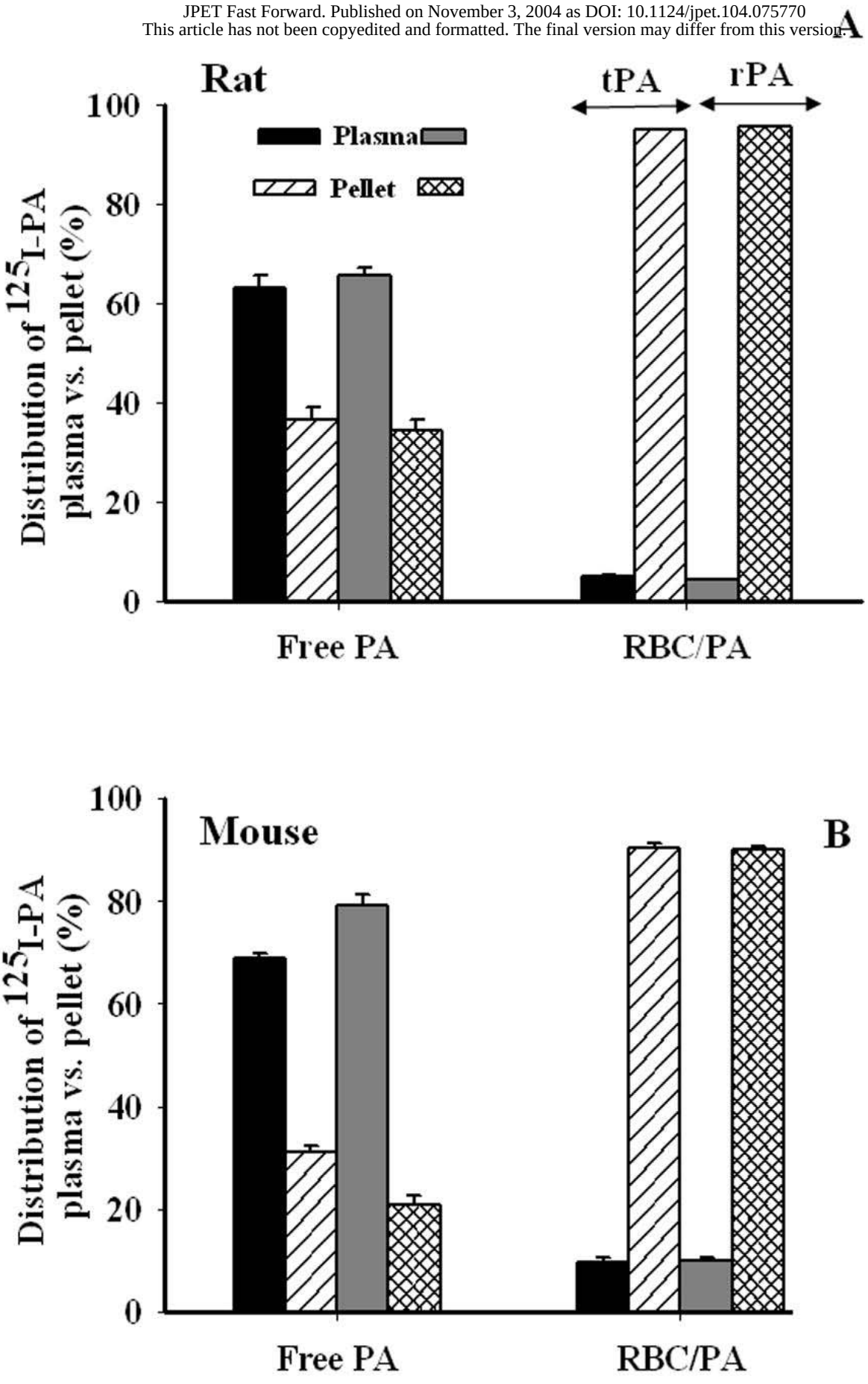

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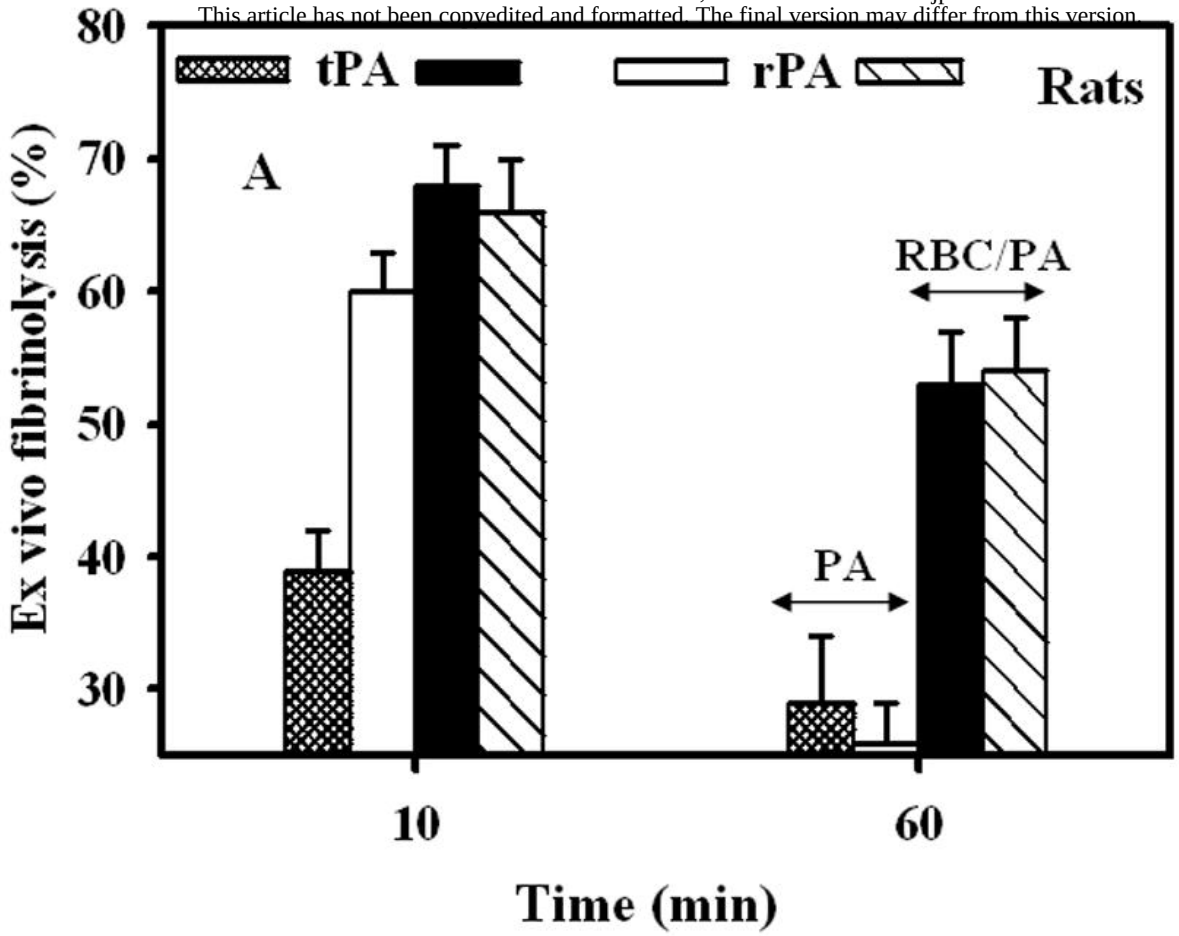

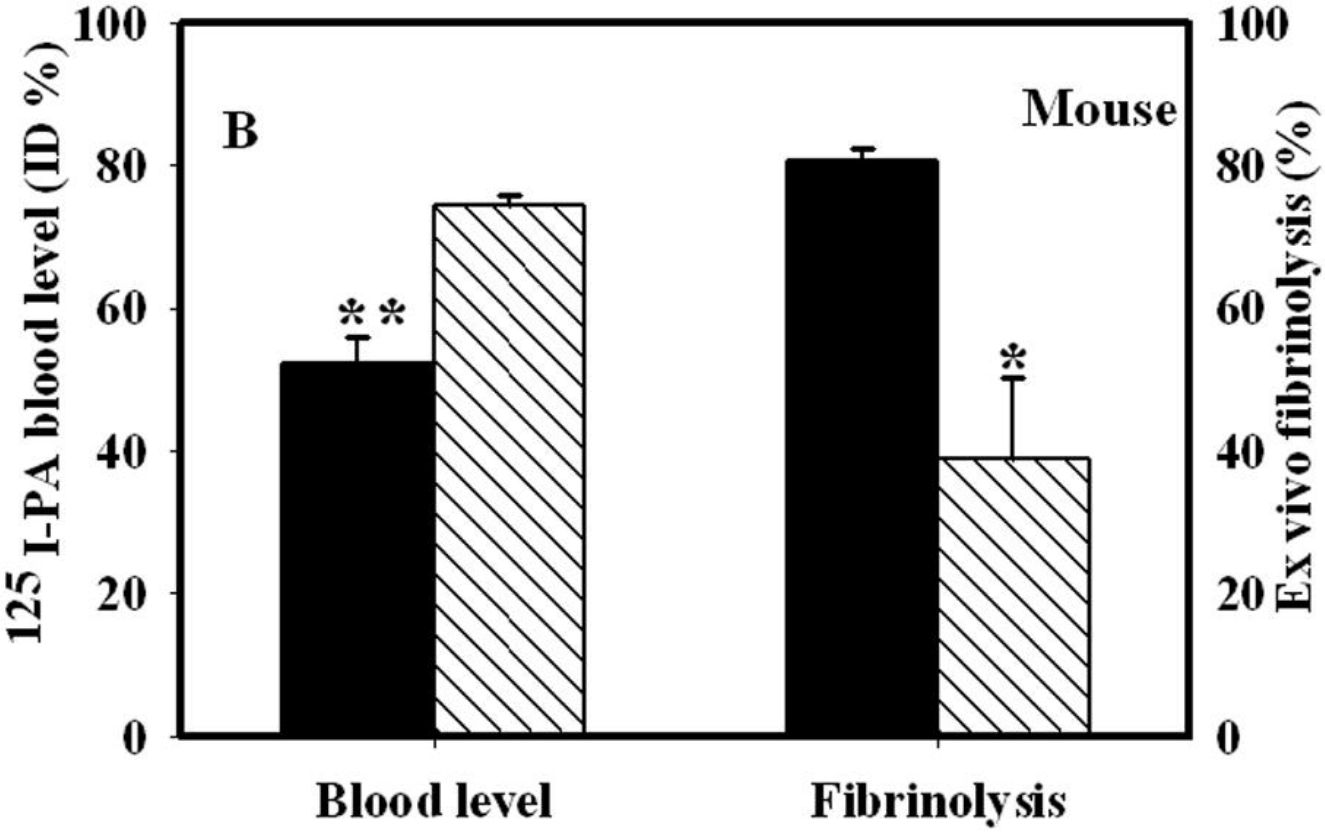

Fig 5

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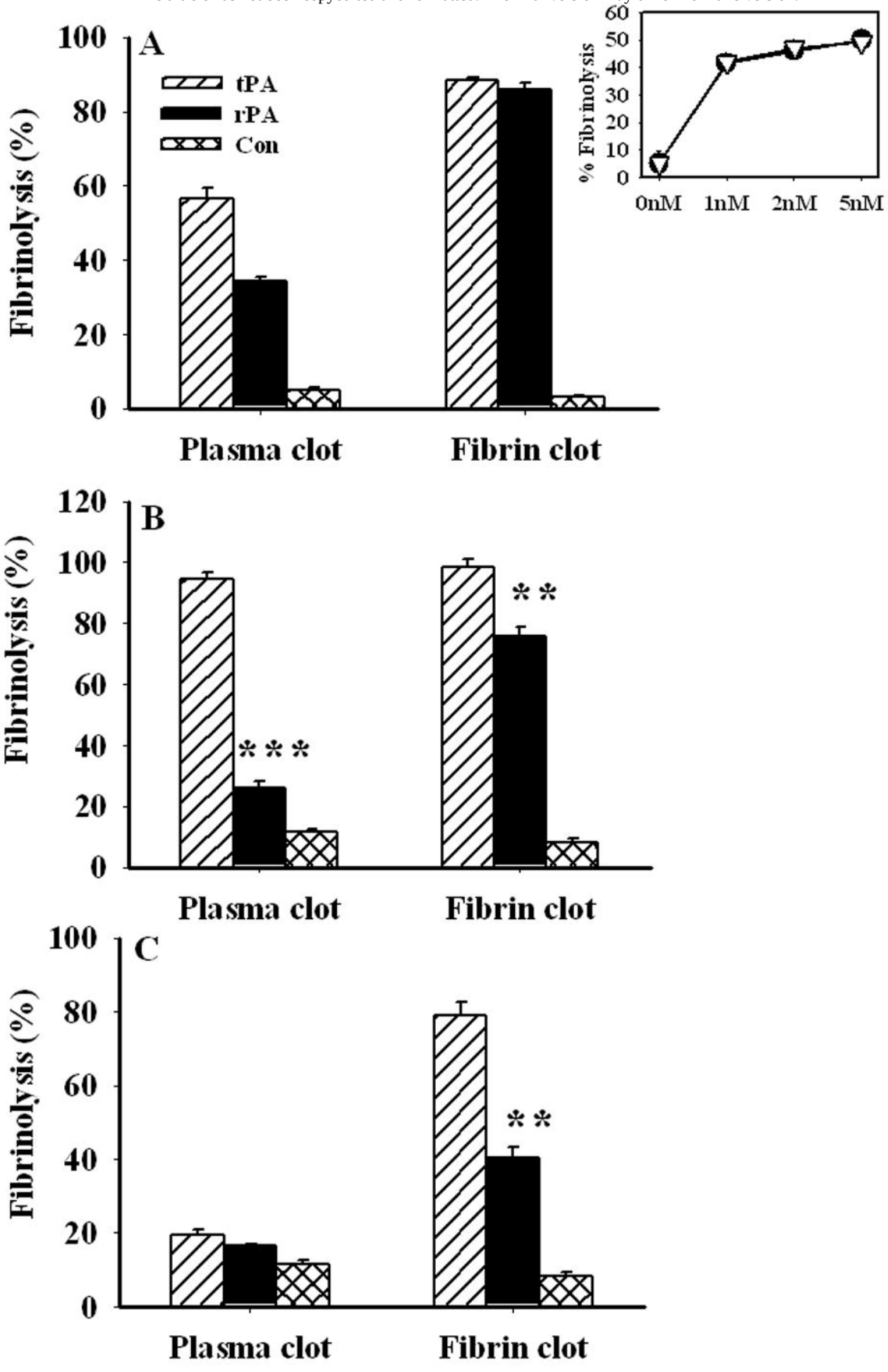

Fig 6

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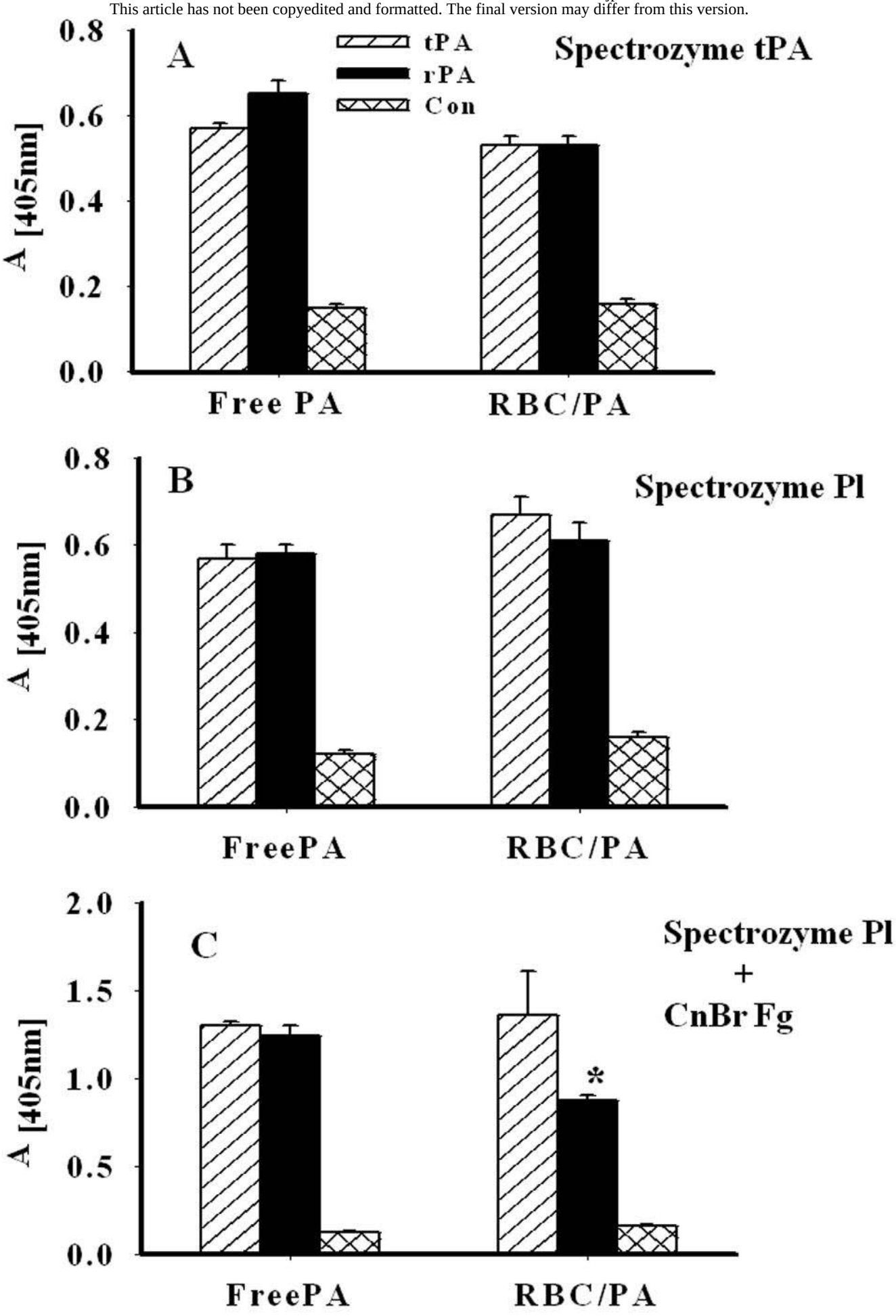

Fig 7

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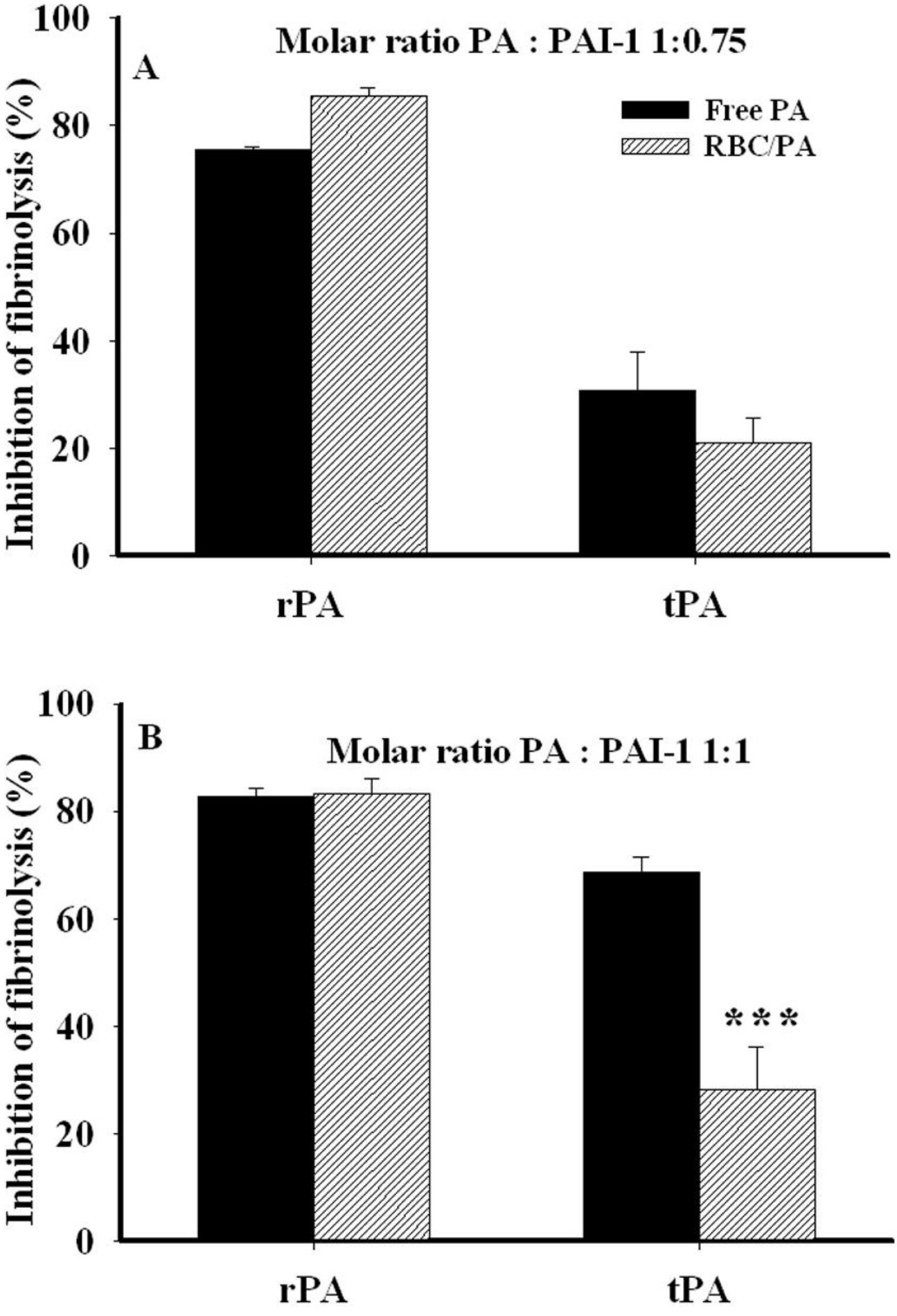

Fig 8