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The glycocalyx protects erythrocyte-bound tissue type plasminogen activator from enzymatic inhibition.

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Abstract

Coupling tissue type plasminogen activator (tPA) to carrier red blood cells (RBC) prolongs its intravascular life-span and permits its use for thromboprophylaxis. Here, we studied the susceptibility of RBC/tPA to PA inhibitors including plasminogen activator inhibitor-1 (PAI-1) that constrain its activity and may reduce the duration of its effect. Despite lesser spatial and diffusional limitations, soluble tPA was far less effective than RBC/tPA in dissolving clots formed *in vitro* from blood of wild type (WT) mice (40 *vs.* 80% lysis at equal doses of tPA). Further, after IV injection, soluble tPA lost activity faster in transgenic mice expressing high level of PAI-1 than in wild type (WT) mice, whereas the activity of RBC/tPA was unaffected. PAI-1 inactivated soluble tPA at equimolar ratios *in vitro*, but had no effect on the amidolytic or fibrinolytic activity of RBC/tPA. RBC/tPA was also more resistant than soluble tPA to *in vitro* inhibition by other serpins (α_2-macroglobulin (α_2M), α_1-anti-trypsin (α_1AT)) and pathologically high levels of glucose. However, coupling to RBC did not protect a truncated tPA mutant, Retavase, from plasma inhibitors. Chemical removal of the RBC glycocalyx negated tPA protection from inhibitors: tPA coupled to glycocalyx-stripped RBC bound twice as much ^{125}I-PAI-1 as did tPA coupled to naïve RBC and susceptibility of the bound tPA to inhibition by PAI-1 was restored. Thus, the RBC glycocalyx protects RBC-coupled tPA against inhibition. Resistance to high levels of inhibitors *in vivo* contributes to the potential utility of RBC/tPA for thromboprophylaxis.

There is a need for improved treatment and prevention of thrombosis, especially in patients identified as being at high-risk for recurrence. Thromboprophylaxis by anticoagulants and platelet inhibitors has been modestly successful, but most patients with myocardial infarction and stroke remain unprotected (Lam et al., 1991; Topol et al., 1999; Jackson et al., 2000; Hennan et al., 2002; Hong et al., 2003; Lange and Hillis, 2004). Both unfavorable pharmacokinetics and low benefit/risk ratio has precluded the use of plasminogen activators (PAs) in clot prevention. The effectiveness of PAs is limited by clot penetration and their rapid inactivation by plasma inhibitors and clearance from the blood (Verstraete et al., 1985; Lucore and Sobel, 1988; Thomas et al., 1993; Narita et al., 1995; Rijken et al., 2004). Treatment requires administration of PAs in doses several orders of magnitude above physiological levels to attain therapeutic efficacy, posing a considerable risk of hemorrhage (Ridker et al., 1994). To date, modifications in the design and delivery of plasminogen activators to attain greater affinity for fibrin, longer circulation time and greater resistance to inhibitors have been of rather modest clinical benefit (Keyt et al., 1994; Benedict et al., 1995; Liberatore et al., 2003; Rijken et al., 2004; Inoue et al., 2005).

To be of use in thromboprophylaxis, a fibrinolytic agent should selectively lyse potentially occlusive clots during their formation, without affecting hemostatic clots or exerting extravascular toxicity (Wang et al., 1998; Melchor and Strickland, 2005). Moreover, to be practical, delivery must be feasible and PA activity must be expressed for hours to days. However, all existing fibrinolytics are relatively short-lived (<30 min) and small (<10 nm diameter) agents, capable of diffusion into hemostatic clots and the

tissues, including the CNS. None can be used as prophylaxis, even in the patients known to be at high risk of imminent or recurrent thrombosis.

Prior studies showed that coupling tPA to red blood cells (RBC) produces long-circulating enzymatically active complexes, thus converting tPA from a therapeutic agent with considerable safety concerns into an effective and safe thromboprophylactic agent (Murciano et al., 2003; Ganguly et al., 2005; Ganguly et al., 2006). Injection of either preformed RBC/tPA (Murciano et al., 2003; Ganguly et al., 2005; Ganguly et al., 2006) or tPA derivatives targeted to complement receptor-1 on circulating RBC (Zaitsev et al., 2006) prevents subsequent formation of occlusive venous and arterial clots, with no overt harmful effects on the carrier RBC (Murciano et al., 2003; Ganguly et al., 2005; Ganguly et al., 2006), activation of coagulation (Murciano et al., 2003) or impaired post-surgical hemostasis (Zaitsev et al., 2006).

Of particular interest, previous studies showed that RBC/tPA lysed human plasma clots more effectively than soluble tPA added to the clots at equal doses despite the obvious diffusional/spatial disadvantage of RBC/tPA (Ganguly et al., 2005). One possible explanation for this surprising result is that coupling to RBC renders tPA less susceptible to plasma PA inhibitors, including the most physiologically relevant, plasminogen activator inhibitor 1 (PAI-1) (Loskutoff et al., 1989). Sensitivity to inhibitors is one of the factors that may control the longevity of RBC/tPA activity *in vivo* and, therefore, the durability of thromboprophylaxis. Plasma levels of PAI-1 are several-fold higher than tPA in healthy subjects and rise further in response to platelet activation (Potter van Loon et al., 1992; Zhu et al., 1999) and vascular injury (Schneiderman et al., 1992; Cesari and Rossi, 1999). High levels of PAI-1 contribute to the resistance of

arterial clots (Potter van Loon et al., 1992; Zhu et al., 1999) and pulmonary emboli to therapeutic lysis (Chapman et al., 1990). In this study we investigated the mechanism by which tPA coupled to RBC may be protected against inhibitors.

Materials and Methods

Materials: Human recombinant tPA (Alteplase) was from Genentech (South San Francisco, CA) and rPA (Reteplase) was from Centocor Inc (Horsham, PA); plasminogen, (Pg) spectrozyme-tPA and PAI-1 were from American Diagnostica (Greenwich, CT); streptavidin (SA), α_2-macroglobulin and α_1-anti-trypsin were from Calbiochem (San Diego, CA); human thrombin, neuraminidase, hyaluronidase and heparinase were from Sigma-Aldrich (St Louis MO); human fibrinogen was from Enzyme Research Laboratories Inc (South Bend IN); iodogen and long-chain 6-biotinylaminocaproic acid N-hydroxysuccinimide ester (B-LC-NHS) were from Pierce Chemical (Rockford, IL). Non-cleavable human plasminogen (NC-Pg [R561A]; hereafter NC-Pg) was expressed in Drosophila S2 cells stably transfected with cDNA encoding a plasmin resistant mutant of plasminogen (a kind gift from Dr. Castellino, University of Notre Dame, South Bend, IN). Proteins were radiolabeled with Na(^{125}I) (Perkin-Elmer Life and Analytical Sciences, Boston, MA) using Iodogen according to the manufacturer's instructions. Free ^{125}I was removed using a Biospin 6 column (Bio-Rad, Hercules, CA). Wild type (WT, C57BL/6J) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Transgenic mice overexpressing a murine PAI-1 minigene (PAI-1Tg$^+$) under control of a CMV promoter were generated as described (Eitzman et al., 1996) and crossed greater than 20 generations to C57BL/6J were the kind gifts of Dr. Ginsburg, University of Michigan (Ann Arbor, MI).

Coupling of tPA to carrier RBC: RBCs were isolated by centrifugation from fresh anti-coagulated human and mouse blood. Approximately 6x10^4 molecules of tPA were attached per RBC without loss of enzymatic activity or alterations in RBC

biocompatibility as previously described (Muzykantov and Murciano, 1996; Murciano et al., 2003; Ganguly et al., 2005; Ganguly et al., 2006). Briefly tPA and RBCs (10% Hct) were biotinylated with B-LC-NHS at 5 (b_5tPA) and 10 (b_{10}RBC) fold molar excess. Biotinylated RBCs were mixed with naive RBCs (1:1) to prevent agglutination and then incubated with SA (10^6 molecules/ RBC). Unbound SA was removed by washing the cells 3 times followed by incubation with radiolabeled b_5tPA. Unbound tPA was removed by washing and cell-associated tPA was measured in a gamma counter (Perkin-Elmer) (Murciano et al., 2003; Ganguly et al., 2005; Ganguly et al., 2006). The b_{10}RBC/b_5tPA conjugates are henceforth designated as RBC/tPA. In separate experiments, RBCs were incubated for 3h at 37ºC with a mixture of glycocalyx-degrading enzymes: neuraminidase (5mU/ml), hyaluronidase (5U/ml) and heparinase (5U/ml) prior to biotinylation. Cells were washed 3 times to remove excess enzyme, biotinylated and incubated with SA and b_5tPA as described above.

***In vitro* lysis of blood clots obtained from mice:** Blood was drawn from WT mice in borosilicate tubes without anticoagulant, ^{125}I-Fg (2µl) and either soluble tPA or coupled to RBC (RBC/tPA) (10nM each) was added and incubated for 20 min at R/T. The release of soluble ^{125}I-fibrin fragments into the supernatant over the next 6 h at 37ºC was measured in a gamma counter (Murciano et al., 2003; Ganguly et al., 2005).

***Ex vivo* fibrinolytic activity of RBC/tPA *vs.* soluble tPA circulating in wild type and PAI-1 overexpressing transgenic mice.** Wild type (C57BL/6J) and PAI-1 Tg^+ mice were divided into two groups and injected with either soluble tPA (0.5mg/Kg) or RBC/tPA (0.2mg/Kg) via the jugular vein. Blood (200µl aliquots) was drawn 5 min and 15 min post-injection into borosilicate tubes without anti-coagulant and 2µl of ^{125}I-Fg

was added immediately. The blood was allowed to clot over the next 20 min at room temperature and fibrinolysis was determined by release of radiolabeled fibrin degradation products as described above (Murciano et al., 2003; Ganguly et al., 2005; Ganguly et al., 2006).

Amidolytic activity of soluble PA and RBC/PA in the presence of PAI-1: Soluble tPA, rPA, RBC/tPA and RBC/rPA (0.05 µM) were pre-incubated with an equimolar concentration of PAI-1 for 30 min at room temperature followed by incubation with Spectrozyme tPA (0.4mM). The preparations were incubated with Spectrozyme (final volume 200µl) in V shaped plates for 20 min at 25ºC, centrifuged at 1200g for 2 min to precipitate RBC and the optical density was measured at 405nm in 100 µl aliquots of the supernatants (Ganguly et al., 2005).

Effect of plasma PA inhibitors on lysis of fibrin clots by soluble PA and RBC/PA. Soluble and RBC/PA (both tPA and rPA) (5nM) were pre-incubated with PAI-1, α_2-macroglobulin and α_1-anti-trypsin in varying molar ratios (1.0:0.5-4.0) for 30 min at room temperature. Fibrin clots were prepared by adding a trace amount of ^{125}I-Fg (2µl) to a 3mg/ml human fibrinogen containing plasminogen. $CaCl_2$ and thrombin (final concentrations 20mM and 0.2U/ml, respectively) were added and PBS (200µl) was layered over the clots. Fibrinolytic agents were added to the clots to simulate their proposed *in vivo* application (Murciano et al., 2003; Ganguly et al., 2005; Ganguly et al., 2006). Soluble tPA mixed in PBS (200µl) was added externally onto the clot surface to model therapeutic dissolution of preexisting clots, while RBC/tPA was incorporated into the clot to model prophylactic lysis of nascent clots (Ganguly et al., 2005). Clot lysis was measured by the release of the soluble ^{125}I-fibrin in the supernatant.

Binding of ^{125}I-PAI-1 to tPA coupled to naïve or glycocalyx stripped RBC: RBC/tPA conjugates prepared using naïve or glycocalyx stripped RBC were incubated with ^{125}I-PAI-1 at a 1:1 molar ratio relative to RBC-coupled tPA for 30 min at room temperature. Unbound ^{125}I-PAI-1 was removed by washing and RBC-bound radioactivity was used to calculate the number of PAI-1 molecules bound to RBC/tPA.

Binding of RBC/PA and glycocalyx stripped RBC/PA to plasminogen. We measured binding of RBC/PA to immobilized proteins as previously described (Ganguly 2005). Plasminogen (Pg) and NC-Pg (0.2 μM/well) were immobilized on 24 wells polystyrene plate overnight at 4ºC. Protein-coated wells were blocked with PBS-BSA (3%) and incubated with RBC/PA for 20 min at room temperature on a horizontal shaker at a low speed (2g). In a separate series, RBC/PA was incubated in Pg-coated wells in the presence of 0.1M EACA, a lysine analog which blocked the lysine-binding sites on immobilized Pg. Binding of RBC was quantified by measuring hemoglobin at 405 nm after lysing the bound cells in water as describe earlier (Ganguly et al., 2006).

Fibrinolytic activity of soluble tPA *vs.* RBC/tPA in presence of high level of glucose: Soluble tPA or RBC/tPA were incubated with 0, 120, 200, 400 and 600 mg/dL glucose in PBS-BSA for 24h at 4ºC. Lysis of ^{125}I fibrin clots was determined as described above. In a separate series of experiments, naïve RBC/tPA or glycocalyx stripped RBC/tPA were incubated with glucose prior to addition within fibrin clots.

Results

Coupling to RBC renders tPA less susceptible to plasma inhibitors. The results of prior studies indicated that RBC/tPA cause less extensive lysis of clots prepared from pure fibrinogen than soluble tPA on a molar basis (Ganguly et al., 2005), likely due to limitations on diffusion and spatial freedom of tPA imposed by the carrier RBC. Yet, RBC/tPA caused more extensive *in vitro* lysis of clots formed from human plasma than soluble tPA. These seemingly discrepant observations implied that coupling to RBC in some way enhances the resistance of tPA to plasma inhibitors, such as PAI-1.

We first evaluated the relative resistance of RBC/tPA *vs.* tPA to inhibition *in vivo* by injecting these formulations intravenously in WT and PAI-1 Tg^+ mice. The plasma level of PAI-1 averages <0.8 ng/ml in WT mice *vs.* 30 ng/ml in PAI-1 Tg^+ mice (Eitzman et al., 1996); hence this study also tested whether enhanced resistance of RBC/tPA to PAI-1 is evident in the pathological settings associated with elevated levels of the inhibitor. Blood was drawn, allowed to clot in the presence of ^{125}I-fibrinogen, and clot lysis was determined (Fig. 1). Previous studies showed that the half-life of radiolabeled RBC/tPA in mice is >3 hours *vs.* <15 min for soluble tPA (Ganguly et al., 2005). Therefore, mice were injected with either 0.5 mg/kg soluble tPA or 0.2 mg/kg RBC/tPA and blood was drawn at 5 and at 15 min to compensate for the rapid clearance of soluble tPA.

After a 6 h incubation at 37^{o}C, clots formed from the blood of WT and PAI-1 Tg^+ mice underwent 15.9±0.8% (Fig. 1, dash line baseline) and 12.6±1.3% spontaneous lysis, respectively. Five min after injection of tPA, clot lysis rose to 60.2±2.7% in WT mice *vs.* 33.8±5.6% in PAI-1 Tg^+ mice ($p<0.05$). Within 15 min of injection, soluble tPA

exhibited a markedly decreased fibrinolytic capacity, which remained close to baseline in both WT and PAI Tg+ mice (Fig. 1B). In contrast, RBC/tPA retained very similar activity in WT and PAI-1 Tg^+ mice at both 5 and 15 min after injection (87.9±6.7 *vs.* 75.7±12.4% and 75.19±9.5 *vs*. 63.2±5.2% at 5 and 15 min, respectively). Thus, in contrast to soluble tPA, RBC/tPA is relatively resistant to highly elevated levels of PAI-1 *in vivo*.

In order to exclude the pharmacokinetic effects, we compared lysis by the same concentration (10 nM) of RBC/tPA *vs*. tPA added directly to blood drawn from WT mice prior to clotting *in vitro*. Despite being subject to fewer spatial constraints, soluble tPA caused less than 50% as much fibrinolysis as RBC/tPA in the blood of WT mice (41.1$\pm$9.3 *vs*. 83.3$\pm$2.4%, respectively).

These data: i) affirm the greater fibrinolytic potency of RBC/tPA compared with soluble tPA in mouse blood *in vitro* and *in vivo*; and ii) indicate that coupling to RBC protects tPA against physiologic and pathologic concentrations of PAI-1.

RBC/tPA is less susceptible to diverse PA inhibitors than soluble tPA. We next compared the susceptibility of soluble tPA *vs*. RBC/tPA and soluble rPA *vs.* RBC/rPA to various purified plasma PA inhibitors. Equimolar concentrations of PAI-1 caused almost complete loss of tPA-mediated chromogenic (Fig. 2A) and fibrinolytic (Fig. 2B) activity, whereas neither activity of RBC/tPA was affected under the same experimental conditions (Fig. 2, left panels). However, coupling of rPA to RBC provided little protection of its amidolytic activity (Fig. 2C) and no protection of its fibrinolytic activity (Fig. 2D) from inhibition by PAI-1.

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Similarly, equimolar concentrations of two plasma serpins with broad spectrum of inhibition, α_2-macroglobulin and α_1-antitripsin, suppressed the fibrinolytic activity of soluble tPA, but not RBC/tPA (Fig. 3). Again, coupling of rPA to RBC did not protect its fibrinolytic activity against inhibition by α_2-macroglobulin (53.2$\pm$2.1 and 52.5$\pm$12.7% inhibition of rPA and RBC/rPA, respectively) or α_1-antitripsin (24.9$\pm$8.7 and 48.8$\pm$12.1% inhibition of rPA and RBC/rPA, respectively).

The RBC glycocalyx protects coupled tPA from plasma serpins. The enhanced resistance of RBC-coupled tPA to plasma inhibitors could result from conformational changes in the tPA molecule caused by conjugation and/or its masking by RBC glycocalyx. To study the mechanism in greater detail, we coupled tPA to RBCs that had been pre-incubated with a mixture of glycocalyx-degrading enzymes (heparinase, neuraminidase and hyaluronidase) and tested the susceptibility of the resulting RBC/tPA to serpins. Equimolar amounts of inhibitors were added relative to the amount of RBC-bound tPA determined using ^{125}I-tPA (6$\pm$0.2 x 10^4 and 3.2$\pm$0.1 x 10^4 tPA molecules per naïve and glycocalyx stripped RBC, respectively).

Tissue type plasminogen activator (tPA) coupled to RBC or glycocalyx-stripped RBC exhibited similar amidolytic (e.g., A_{405} equal 0.90$\pm$0.03 in RBC/tPA *vs.*0.91$\pm$0.26 in stripped RBC/tPA in the Spectrozyme assay) and fibrinolytic activity (Fig 4A). However, coupling tPA to glycocalyx-stripped RBC did not confer the enzyme with enhanced resistance to PAI-1, α_2M or α_1AT (Fig. 4A). In support of this inference, binding of ^{125}I-labeled PAI-1 to tPA coupled to glycocalyx-stripped RBC almost doubled compared with binding to tPA coupled to control RBC (Fig. 4B). These data suggest that the RBC glycocalyx protects cell-bound tPA against plasma serpins by masking their

auxiliary binding sites (Carrell et al., 1991; Lawrence et al., 1995; Wilczynska et al., 1995).

Stripping of RBC glycocalyx does not affect the binding of RBC/tPA to plasminogen. We next tested whether RBC glycocalyx also affects the interaction of RBC-bound tPA with its physiological substrate, plasminogen. RBC/tPA, but not RBC/rPA bound to plastic wells coated with Pg (Fig. 5). Naïve RBC showed no binding to Pg (Fig. 5, dash line), confirming specificity of RBC/tPA binding. Further, RBC/tPA showed no binding in albumin-coated wells (Fig. 5, inset). RBC/tPA binding to Pg was blocked by the lysine analog i.e, EACA or by replacing Pg with its non-cleavable analogue, NC-Pg (Fig. 5), indicating that binding requires interactions between *lys* binding sites in plasminogen activators and *lys* residues in the substrate. However, RBC/tPA and stripped RBC/tPA bound to plasminogen to a similar extent ($28.3\pm3.1 \times 10^3$ *vs.* $25.2\pm4.9 \times 10^3$ RBCs/well, respectively).

Coupling to RBC protects tPA against inhibition by high levels of glucose. Pathologically high levels of glucose have also been shown to inhibit tPA activity (Alvarez-Sabin et al., 2004). Therefore, we next tested whether coupling to RBC alters tPA's susceptibility to elevated levels of glucose. Addition of pathologically high levels of glucose (600 mg/dL) directly to fibrin clots along with tPA did not affect either spontaneous lysis or lysis induced by RBC/tPA or soluble tPA (inset in Fig. 6A). This data corroborates findings that tPA inhibition via non-enzymatic glycosylation/glycation requires a relatively prolonged (i.e., hours) exposure (Lapolla et al., 2005).

Pre-incubation with glucose at high concentrations for 24 hours markedly inhibited the fibrinolytic activity of soluble tPA, but not RBC/tPA (Fig. 6A). Further, tPA

coupled to glycocalyx-stripped RBC was less resistant to the inhibitory effect of glycation than tPA coupled to naïve control RBC (Fig. 6B). This data implies that, in addition to masking binding sites for protein inhibitors (Fig. 4B); the RBC glycocalyx exerts a more general stabilizing effect on tPA molecule.

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Discussion

The fibrinolytic activity of tPA in plasma is regulated primarily by diffusion-limited inactivation by the suicide substrate PAI-1 and to a lesser extent by other plasma serpins such as α_1-anti-trypsin, α_2-macroglobulin and C1 inhibitor (Madison et al., 1990; Chandler et al., 1995). PAI-1 traps tPA in the form of an inactive stabilized acyl enzyme intermediate (1:1 complex) (Carrell et al., 1991; Lawrence et al., 1995) that is cleared from plasma with a half-life of 3-5 min by the low-density lipoprotein related receptor and the VLDL-receptor (Bu et al., 1992) leading to degradation of the enzyme in lysosomes. Increased expression of PAI-1 by ischemic endothelium and release from activated platelets contributes to the resistance of platelet rich arterial thrombi to lysis (Zhu et al., 1999).

In part to compensate for rapid inactivation and clearance, tPA must be given at concentrations several orders of magnitude higher than physiological concentrations to mediate reperfusion after myocardial infarction or stroke (Ridker et al., 1994). The need for such high doses for efficacy is accompanied by a considerable risk of hemorrhage. Genetic reengineering of tPA to remove critical auxiliary sites that help mediate serpin binding (amino acids 296-304) increases the molecule's resistance to PAI-1 (Madison et al., 1990) and prolongs its circulation in plasma (e.g., TNKase mutant) (Keyt et al., 1994), but not to the extent needed to provide thromboprophylaxis. Moreover, TNKase, like other tPA variants, is a relatively small (~60 kD) protein capable of diffusing into pre-existing hemostatic clots and through blood-brain-barrier perturbed by ischemia into brain parenchyma, hence increasing the risk of hemorrhages and other adverse effects.

Results of the previous studies have shown that coupling tPA to carrier RBC increases its intravascular life-span by several orders of magnitude compared with soluble tPA (Murciano et al., 2003; Ganguly et al., 2005) and restricts PA extravasation into brain (Murciano et al., 2003). During the course of these studies, RBC/tPA was found to be less active than soluble tPA in lysing clots formed from fibrinogen, likely due to steric constraints on permeation, but showed clear fibrin specificity (Ganguly et al., 2005) and, surprisingly, was more active than soluble tPA in lysing clots made from human plasma (Ganguly et al., 2005). These findings suggested a fundamental change in the relationship between tPA-mediated plasminogen activation and inhibition by cognate serpins that results from coupling the enzyme to the cell surface.

Studies in the present paper show that the protection afforded by RBC binding is dependent on the cell's glycocalyx. Enzymatic degradation of the glycocalyx did not affect basal amidolytic (0.90±0.03 in RBC/tPA *vs.*0.91±0.26 in stripped RBC/tPA) or fibrinolytic activity of RBC-coupled tPA (Fig 4A). However, removal of the glycocalyx led to the loss of tPA's protection by RBC, both in terms of PAI-1 binding and tPA activity. These results do not exclude that a change in tPA's conformation imposed by conjugation contribute to protection, but favor a more specific effect of the glycocalyx on PAI-1 binding to the auxiliary sites on tPA, access to tPA catalytic site or restrictions on conformational changes in PAI-1 that occur after binding (Carrell et al., 1991; Lawrence et al., 1995; Wilczynska et al., 1995; Egelund et al., 2001).

The RBC glycocalyx affects the interaction of RBC-bound tPA with its inhibitors, activators (e.g., fibrin) and substrates (physiological substrate, plasminogen and small synthetic substrates used to measure amidolytic activity) in a complex, but precise way.

Our previous studies showed that coupling to RBC does not affect the stimulation of tPA activation by fibrin (Ganguly 2005). The results of the present study indicate that the RBC glycocalyx does not affect RBC/tPA binding to plasminogen (Fig. 5). Therefore, it appears that coupling to RBC does not compromise tPA interactions with its substrates and activators, yet protects it from the inhibitors.

It is known that cellular glycocalyx may affect the activity of plasmin (Pluskota et al., 2004). Therefore, in theory, the enhanced activity of RBC/tPA seen in the presence of inhibitors could be due to protection of plasmin. However, coupling to RBC did not protect a truncated form of tPA, rPA, against plasma inhibitors (Fig. 2). This selectivity argues for a direct effect of the RBC glycocalyx on tPA. This interpretation is supported by the fact that the RBC glycocalyx protects the amidolytic activity of tPA as well.

It is not clear yet why RBC does not protect rPA. In theory, this result can be explained by: i) lack of auxiliary sites for interactions with glycocalyx or/and inhibitors on the rPA molecule which lacks the finger, growth factor and Kringle-1 domains present in tPA; and/or, ii) unique conformational changes caused in rPA is a result of conjugation. It is an interesting corollary in this context that despite the fact that RBC/tPA and RBC/rPA possess equal activity in the absence of inhibitors (indicating that they interact equally well with plasminogen and convert it to plasmin), only RBC/tPA possess sufficient affinity to plasminogen to afford specific binding via lysine-mediated mechanism (Fig. 5).

Understanding the mechanism by which the glycocalyx protects tPA from PAI-1 and other serpins will require additional studies. The most simplistic explanation that the glycocalyx physically masks the auxiliary binding site(s) for PAI-1 seems less likely

based on the selective effect in this tPA feature with no effect on of the enzyme access to plasminogen or fibrin. Rather, specific "protection" of tPA by RBC glycocalyx against serpins may be explained by interference with charge-mediated interactions between the protease and protein inhibitor. It appears likely that PAI-1 contacts with the surface loop of tPA via residues 350-355, which contains several negatively charged amino acids (Glu-Glu-Ile-ILe-Met-Asp) (Madison et al., 1990). The interaction of tPA and PAI-1 may therefore be stabilized through salt bridges formed between cationic amino acids tPA and anionic residues in PAI-1 (Madison et al., 1990; Egelund et al., 2001). Negatively charged components of RBC glycocalyx such as ionized sialic acid groups may impede these interactions by forming an electrostatic as well as a physical barrier between protease and serpins.

The observation that the RBC glycocalyx also protects tPA against inactivation by non-enzymatic glycosylation may support the notion that protection is due to charge-mediated masking of vulnerable sites on tPA molecules by the glycocalyx. Interactions between RBC/tPA and the glycocalyx may mask the lysine residues in tPA molecules that are vulnerable to Maillard product formation and glycation (Lapolla et al., 2005) .

The finding that RBC-bound tPA is more resistant to PAI-1 and other serpins and high levels of glucose translates into additional practical advantages for this modality. These include reduction in the effective dose with attendant lessening of the risk of hemorrhage, extended duration of thromboprophylaxis, enhanced effectiveness towards platelet-rich arterial clots and prevention of thrombosis in diabetics. The results of this study also reveal a relatively unappreciated role for the glycocalyx in modulating tPA

activity, which may take place on the surface of endothelial, hematopoietic and other vascular cell types, that deserves greater scrutiny.

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Footnotes:

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Legends for Figures:

Figure 1: RBC/tPA is more resistant in vivo to PAI-1 than tPA. WT (black bars) and PAI-1Tg^{+} (hatched bars) were given 0.5 mg/kg soluble tPA or 0.2 mg/kg RBC/tPA iv. Blood was taken 5 or 15 min later. Whole blood clots trace labeled with ^{125}I-fibrinogen were allowed to form in non-anticoagulated tubes and the amount of soluble ^{125}I-fibrin degradation products released into the supernatant fluid 6 h later was measured. The dashed line shows spontaneous lysis. Data are shown as mean ± S.D (n=3-4 animals). *p< 0.05.

Figure 2: Coupling to RBC protects both amidolytic and fibrinolytic activity of tPA, but not rPA from inhibition by PAI-1. Soluble (black bars) PA or RBC/PA (hatched bars) (5 nM each) was incubated in PBS or PBS supplemented with an equimolar amount of PAI-1 for 30 min at RT. Panels A and B: tPA, panels C and D: rPA. Panels A and C: Bars show amidolytic activity after addition of Spectrozyme tPA. Panels B and D: *In vitro* fibrin clots were formed using ^{125}I-fibrinogen by the addition of $CaCl_2$ and thrombin. Fibrinolysis was measured as in the legend to Figure 1. The data are shown as mean ± S.D (n=3). ***p<0.001 and **p<0.01.

Figure 3: RBC/tPA activity is resistant to diverse plasma inhibitors. Soluble tPA or RBC/tPA (closed *vs.* open circles) was incubated for 30 min at room temperature with the

indicated molar ratios of PAI-1 (A), α_2-macroglobulin (B) and α_1-antitrypsin (C) prior to measuring ^{125}I fibrin clot lysis as described in Fig 2. Soluble tPA vs. RBC/tPA: *p<0.05 and **p<0.01. Data represents mean ± S.D (n=3).

Figure 4: The RBC glycocalyx protects RBC/tPA from plasma inhibitors. RBCs were incubated with a cocktail of neuraminidase, hyaluronidase and heparinase to remove the glycocalyx and then conjugated with tPA. Two RBC/tPA formulations were tested: tPA coupled to naïve RBC (RBC/tPA, black bars) and tPA coupled to the glycocalyx stripped RBC (stripped RBC/tPA, hatched bars). Both types of RBC/tPA complexes were incubated with PAI-1(1:1), α_2-macroglobulin (1:2) or α_1-antitrypsin (1:2) at indicated molar ratios prior to incorporation within fibrin clots. **Panel A**: Effect of inhibitors on lysis of ^{125}I fibrin clots. **Panel B**: Binding of ^{125}I-PAI-1 to naïve *vs.* glycocalyx-stripped RBC/tPA. The RBC/tPA complexes were incubated with ^{125}I-PAI-1 at a 1:1 molar ratio relative to bound tPA for 30 min at room temperature. Unbound ^{125}I-PAI-1 was removed by washing and residual radioactivity measured. The data shown are the mean ± S.D (n=3). Differences between the groups are significant **p<0.01.

Figure 5: Binding of RBC/PA to plasminogen. Naïve RBC (dash line), RBC/tPA (black bars), RBC/rPA (hatched bars) or stripped RBC/tPA (double-hatched bars) were incubated with plasminogen immobilized in a polystyrene well plate on a horizontal shaker for 20 min at RT. After removal of non-bound RBC by washing, binding of RBC was measured by quantifying hemoglobin at 405nm after lysing the cells in water. The data shown as M+SEM, n=4, ***p<0.001 for binding of RBC/tPA to Pg *vs.* all other groups in the main panel. The inset shows binding of RBC/tPA and stripped RBC/tPA to the wells coated with albumin (left bars) or plasminogen (right bars).

Figure 6: The glycocalyx protects RBC-coupled tPA against glycation. **Panel A**: Fibrinolytic activity of soluble (closed circles) and RBC/tPA (open circles) incubated with the indicated concentrations of glucose in PBS-BSA (3%) for 24 h was tested using ^{125}I fibrin clots as described above. The inset shows fibrinolysis by soluble tPA, RBC/tPA and tPA mixed with glucose (600 mg/dL) *vs.* controls. **Panel B**: Fibrinolytic activity of tPA coupled to naïve RBC (open circle) *vs.* tPA coupled to glycocalyx stripped RBC (closed circles) preincubated with the indicated amounts of glucose. *$p<0.05$, **$p<0.01$. Data shown as mean ± S.D (n=3).

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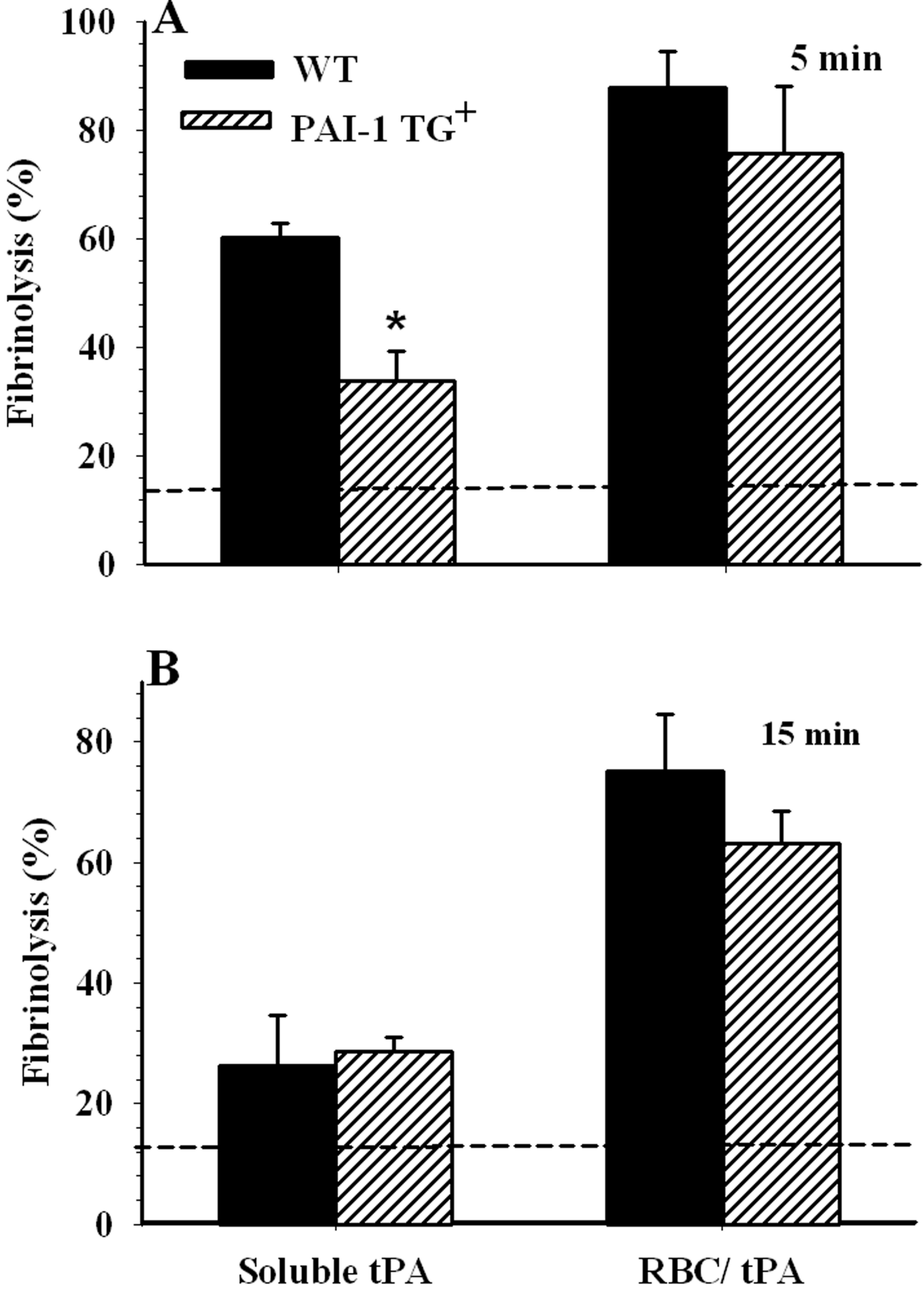

Fig: 1

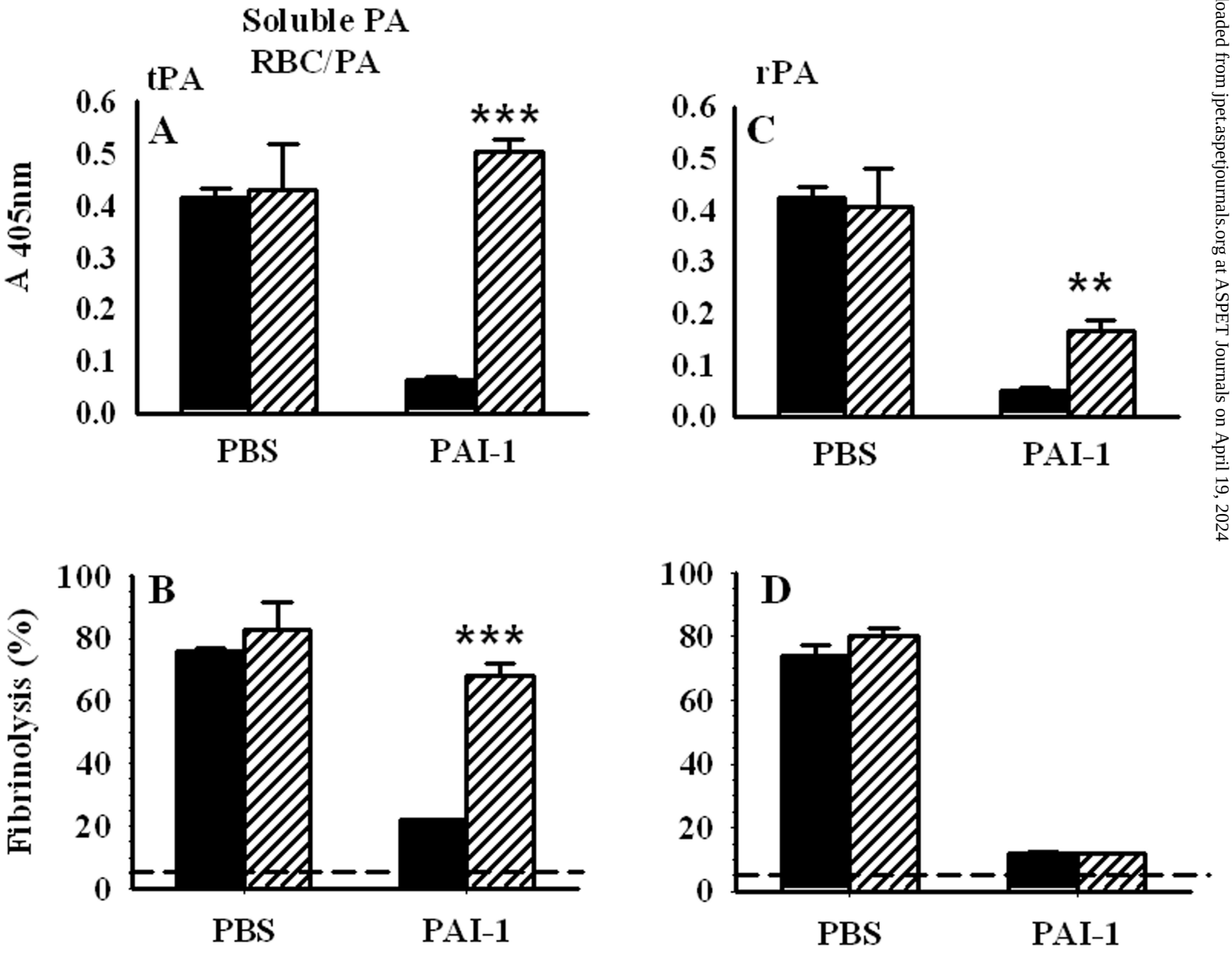

Fig:2

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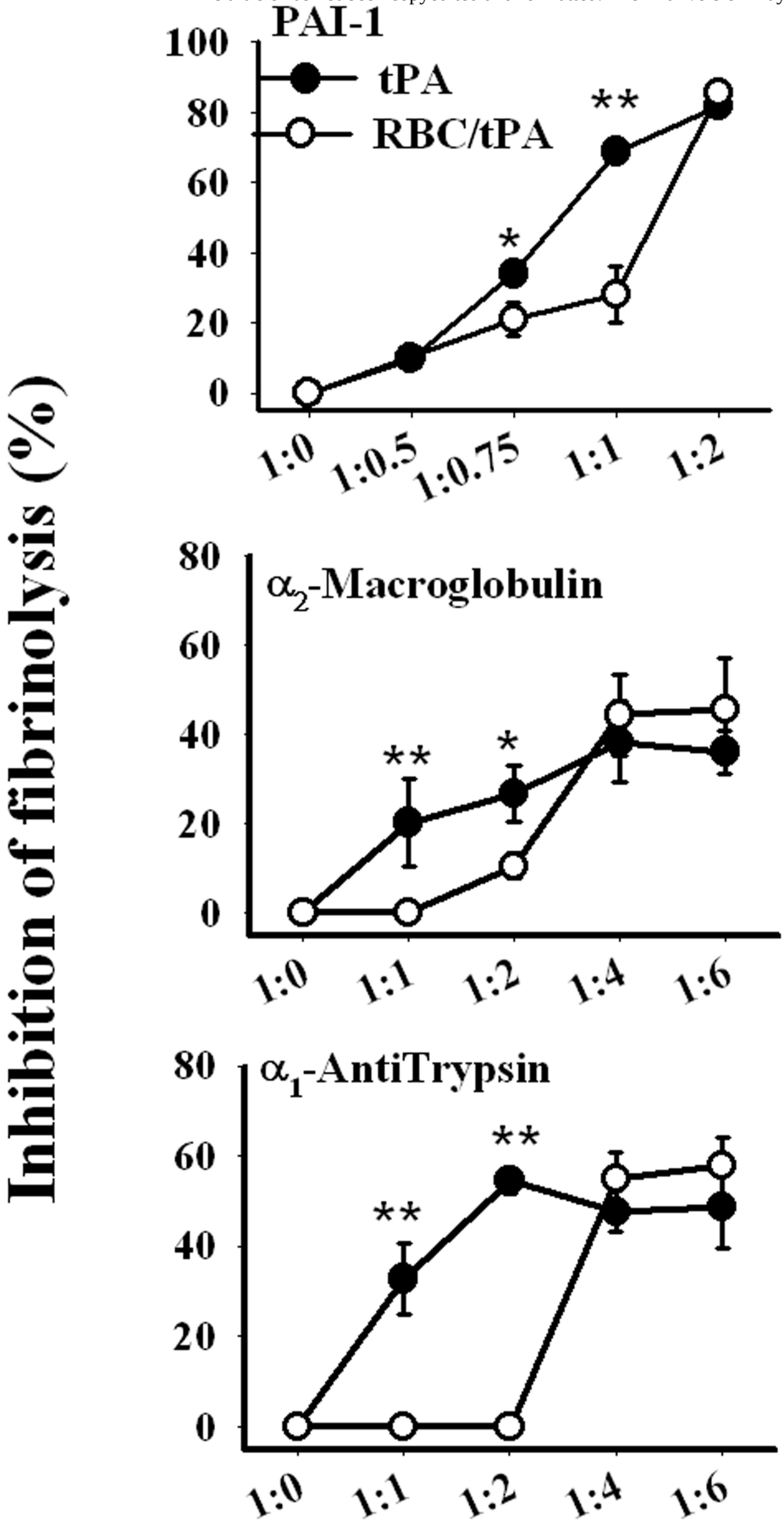

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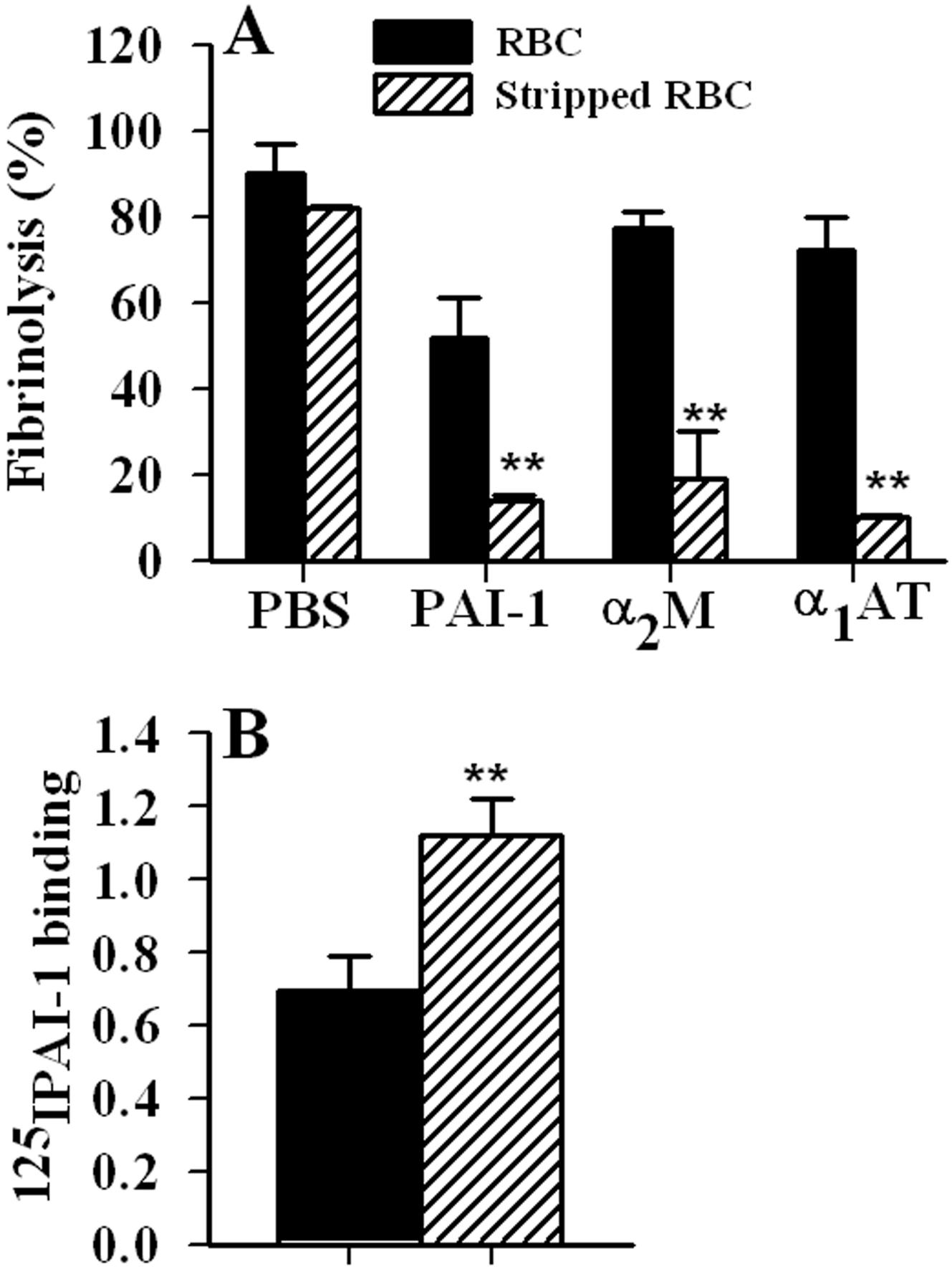

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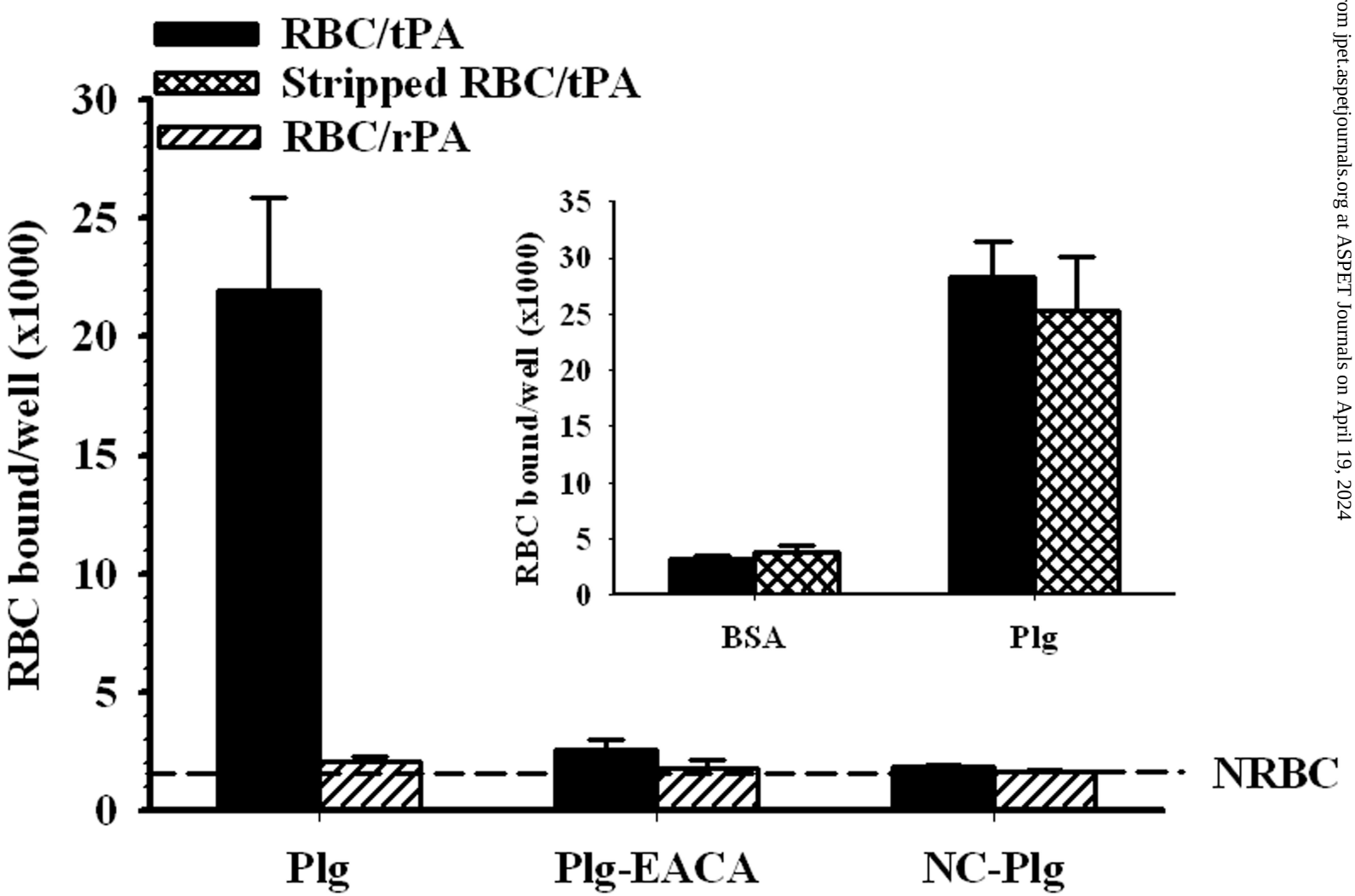

Fig:5

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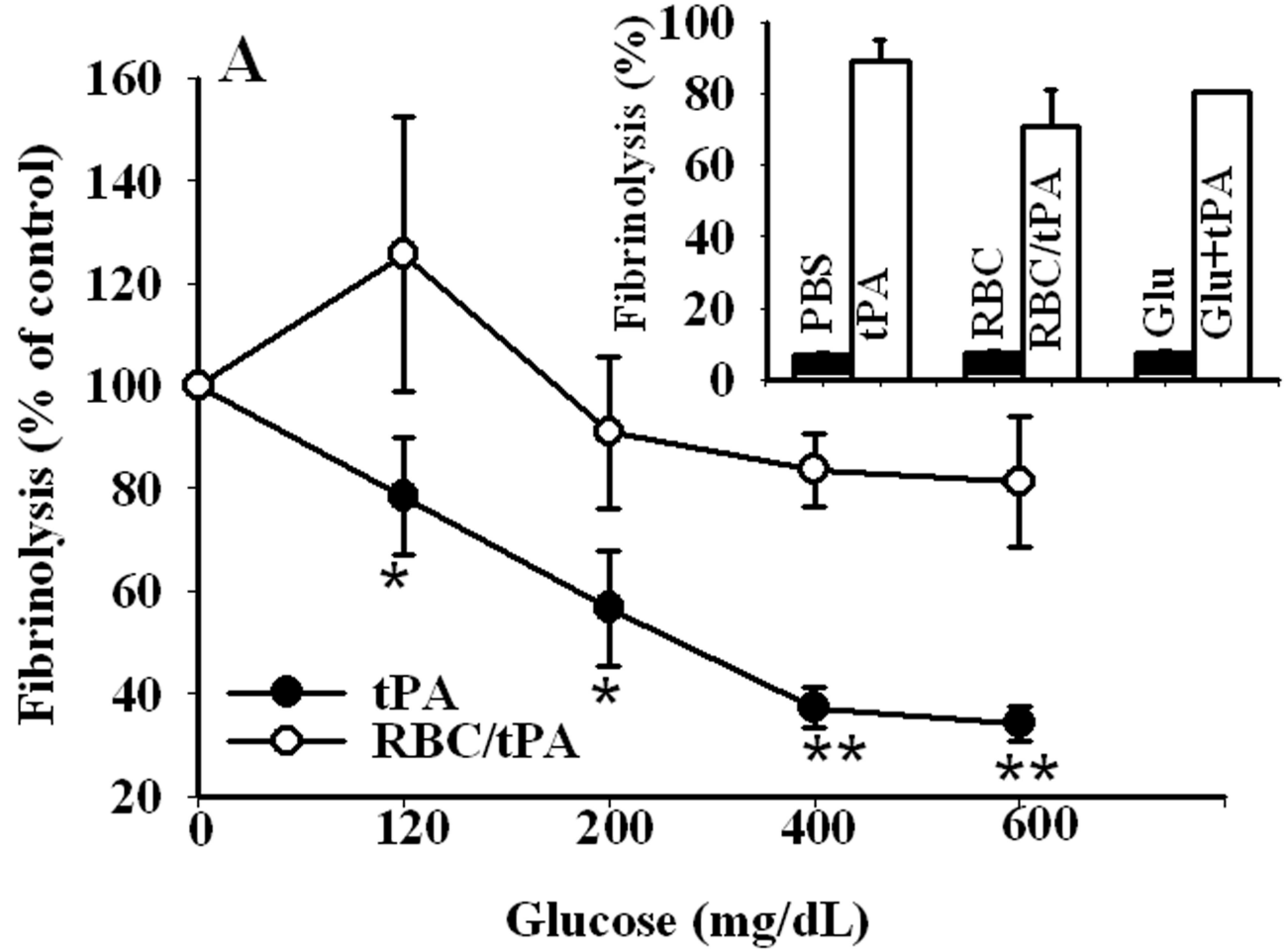

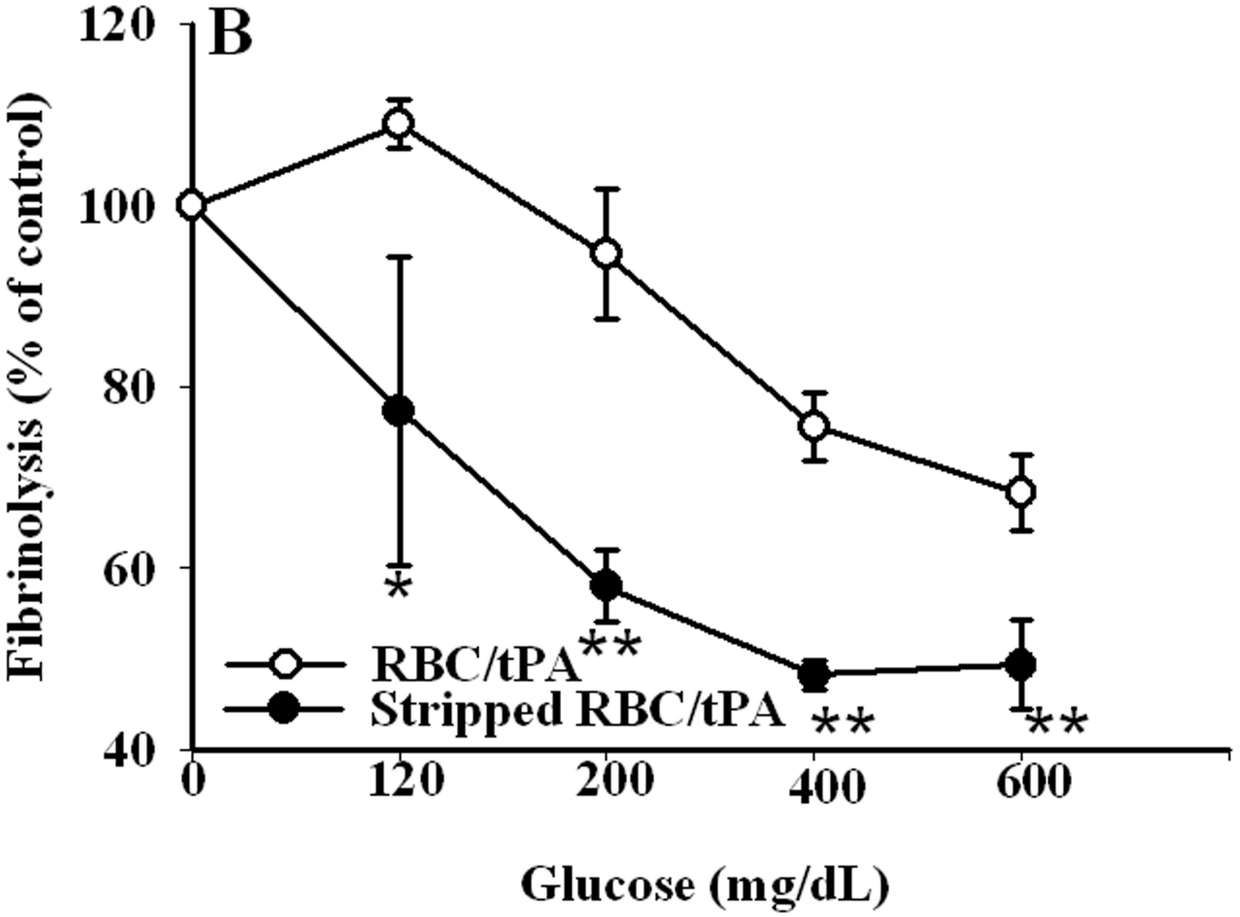

Fig: 6