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Lytic solution of platelet-rich plasma clots potentiated platelet response to agonists by action on Ca^{2+} homeostasis[1]

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ABSTRACT Effects of thrombolytic agents and lytic solution of platelet-rich plasma (PRP) clots (LSPC) on platelet activation as indicated by platelet aggregation, generation of malondialdehyde (MDA), and the concentration of intracellular free calcium ($[Ca^{2+}]_i$) in rats were investigated. Neither urokinase nor streptokinase *in vitro* showed adverse effects on platelet function. The solution of PRP clots incubated either alone (as control) or with urokinase or streptokinase 2000 IU · ml^{-1} at 37℃ for 45 min potentiated the increase of platelet aggregation and MDA formation and produced a persistently high level of $[Ca^{2+}]_i$ stimulated by thrombin, and platelet aggregation induced by ADP. But LSPC had no effects on the Ca^{2+} influx or the release of intracellular stored Ca^{2+}, and no significant difference was found in the promotion of platelet response to agonists between the solution of the clots warmed in the presence or absence of thrombolytic agents. In the thrombosis model in rat abdominal aorta, both urokinase and streptokinase (40 000 IU · kg^{-1}) slightly inhibited electrically stimulated thrombosis. In contrast, LSPC (600 μl · kg^{-1}) considerably enhanced the thrombosis. These findings suggested that the changes of platelet function in ischemic patients receiving thrombolytic therapy may be mediated by the proteolytic products of clots through acting on $[Ca^{2+}]_i$ homeostasis after platelet stimulation rather than by the thrombolytic agents *per se*.

KEY WORDS urokinase; streptokinase; thrombolytic therapy; platelet aggregation; calcium

The beneficial effects of thrombolytic agents in the treatment of intravascular thrombosis is now well established[1]. The thrombolytic agents streptokinase, urokinase, and alteplase (formerly called tissue-type plasminogen activator) offer their beneficial effects by facilitating the direct or indirect activation of plasminogen. In addition to specific thrombolytic actions, the platelet function after treatment with these drugs may change. Alteplase can induce platelet hyperaggregability in rabbits[2]; streptokinase is capable of stimulating the aggregation of platelet in humans[3] and in rabbits[2]. On the other hand, *in vitro* alteplase and urokinase can inhibit agonist-induced aggregation of human platelets[4]. These divergent findings led us to imagine that thrombolytic agents may not be the sole mechanism to affect the platelet response to agonists. Thrombolytic products formed in circulation during the treatment may present another approach to understand the controversial results of these agents on platelet function. In this study, we tested the effects of lysed solution of PRP clots (LSPC) on platelet activation.

MATERIALS AND METHODS

Materials Urokinase (Green Cross Co, Japan); streptokinase, Fura 2-AM, 1, 1, 3, 3-tetraethoxypropane, and ADP as sodium salt (Sigma, USA); porcine thrombin (Biochemical Pharmaceutical Factory of Zhuhai, China).

Platelet Aggregation Fresh blood was obtained from Dark Agouti rats (♀, 280± *s* 63 g) by cardiac puncture. Whole blood was anticoagulated with 3.8% sodium citrate (9 : 1 vol : vol) and centrifuged at 250 × *g* for 10 min to obtain PRP. Platelet-poor plasma (PPP) was prepared by additional centrifugation of the

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whole blood pellet at 1500× *g* for 10 min.

Platelet aggregation was measured with a dual-channel aggregometer (Model DAM-1, Dangyang Institute of Electronics, Jiangsu, China) with 0.45 ml PRP. PRP was prewarmed for 3 min at 37℃ before adding the agent.

MDA formation and measurement PRP was washed twice in isotonic phosphate buffered saline (PBS) solution (pH 7.4, including 0.5 % Na_2EDTA) and centrifuged at 800 × *g* for 10 min. The platelet pellet was finally resuspended in PBS to 2×10^8 · ml^{-1}. Thrombin (final concentration 1 IU · ml^{-1}) was added to the platelet suspension to induce MDA production. After incubation and gentle agitation for 30 min at 37℃, the stimulation was stopped by 50 μl of 8% sodium dodecyl sulfate. Then trichloroacetic acid (40%, 0.4 ml) was added to the suspension and centrifuged at 3000 × *g* for 10 min. The supernatant (1.5 ml) was mixed with 0.5 ml thiobarbituric acid 0.1 mol · L^{-1} and heated at 90℃ for 30 min. 1,1,3,3-tetraethoxypropane was used as a standand to read the fluorescence absorbance at λ_{ex} 515 nm and λ_{em} 553 nm[5].

Measurement of $[Ca^{2+}]_i$ and release of intracellular stored Ca^{2+} platelets were prepared from a single rat[6]. Blood was anticoagulated with acid-citrate-dextrose (6 : 1 vol : vol, citric acid 1.5%, sodium citrate 2.5%, and glucose 2%), and centrifuged at 200 × *g* for 10 min to obtain PRP. After being incubated with 4 μmol · L^{-1} Fura 2-AM for 30 min in a shaking water bath at 37℃, the PRP was centrifuged at 800× *g* for 10 min and the platelet pellet was resuspended in Hepes buffer (containing NaCl 145 mmol · L^{-1}, KCl 5 mmol · L^{-1}, $MgSO_4$ 1 mmol · L^{-1}, Hepes 10 mmol · L^{-1}, pH 7.4, and glucose 10 mmol · L^{-1}) and adjusted to 2×10^8 · ml^{-1}. Platelets were used within 1 h. Two ml of aliquots were preincubated at 37℃ for 3 min in the presence of $CaCl_2$ 1 mmol · L^{-1} before the agents were added. Fluorescence in terms of calcium concentration was measured at 37℃ in 1 cm quartz cuvettes with stirred platelet suspension in a spectrofluorophotometer (Model RF-5000, Shimadzu, Japan) with dual λ_{ex} 340 nm and 380 nm and λ_{em} 510 nm. Calibration of $[Ca^{2+}]_i$ was performed[7].

$$[Ca^{2+}] = K_d(R-R_{min})/(R_{max}-R)\ (S_{f2}/S_{b2})$$

R_{max} was obtained after adding 0.1% Triton X-100 and R_{min} was the ratio in the presence of EGTA (5 mmol · L^{-1}).

Ca^{2+} released from intracellular stores was determined by measuring the increase of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, which was achieved in the presence of EGTA (1 mmol · L^{-1}).

Arterial thrombosis The rectal temperture was taken to evaluate the thrombosis of abdominal aorta initiated by electric stimulation for 7 min with 1.5 mA just beneath the renal arteries. LSPC or agents were injected slowly through tail vein 10 min before the stimulation. The test was carried out at 21—23℃.

PRP clot formation and lysis Thrombin (5 IU) was added to 1 ml of PRP, then gently mixed, and allowed to form clot for 10 min at room temperature. To follow the lysis of PRP clots, urokinase (2000 IU · ml^{-1}), streptokinase (2000 IU · ml^{-1}), or equal volume of PBS was added to the tube and shaked in water bath at 37℃ for 45 min. The tube was centrifuged at 1000 × *g* for 10 min. 50 μl of the supernatant were used in the platelet activation tests.

Statistical methods One way ANOVA and *t* test were used.

RESULTS

Platelet aggregation Effects of thrombolytic agents and LSPC on platelet aggregation were shown in Tab 1. At a concentration of 2000 IU · ml^{-1}, neither urokinase nor streptokinase showed notable effects on platelet aggregation initiated by thrombin (2 IU · ml^{-1}) or ADP (0.2 mmol · L^{-1}). Whereas, 50 μl of the solution of PRP clot lysed alone (SPCLA) or by urokinase (SPCLBU) or streptokinase (SPCLBS) exhibited considerable facilitation to both thrombin- and ADP-induced rat platelet aggregation ($P < 0.01$ *vs* PBS).

MDA formation Effects of thrombolytic agents and LSPC on platelet MDA formation were showed in Tab 1. All of the 3 LSPC markedly potentiated the thrombin-initiated MDA production ($P < 0.01$ *vs* PBS). But the thrombolytic agents exhibited no significant influence on the level of MDA.

Tab 1. Effects of lytic solution of PRP clots (50 μl) and thrombolytic agents (2000 IU · ml^{-1}) on thrombin (2 IU · ml^{-1}) and ADP (2 μmol · L^{-1}) induced platelet aggregation and on malondialdehyde formation in platelet initiated by thrombin (1 IU · ml^{-1}). $n=8$.

$^{*}P>0.05$, $^{***}P<0.01$ *vs* PBS. SK: streptokinase; UK: urokinase; PBS: phosphate buffered saline; solution of PRP clots lysed alone (SPCLA), by streptokinase (SPCLBS), by urokinase (SPCLBU).

	Aggregation/%		Malondialdehyde/
	Thrombin	ADP	nmol·ml^{-1}
PBS	40±6	40±5	0.15±0.05
SK	37±8*	47±9*	0.16±0.07*
UK	35±9*	44±7*	0.17±0.08*
SPCLA	57±7***	61±6***	0.62±0.18***
SPCLBS	65±10***	66±7***	0.74±0.17***
SPCLBU	70±8***	71±11***	0.88±0.16***

$[Ca^{2+}]_i$ Thrombin induced an elevation of $[Ca^{2+}]_i$ from a resting level of 234 ± 48 nmol·L^{-1} to 3.3±0.9 μmol·L^{-1}. In the presence of EGTA, $[Ca^{2+}]_i$ was increased from 242±62 nmol·L^{-1} to 0.9±52 μmol·L^{-1}, which represented the thrombin initiated release of intracellular stored Ca^{2+}. In comparison with the control, SPCLA, SPCLBU, or SPCLBS possessed no potent actions on the amplitude of increased $[Ca^{2+}]_i$ ($P>0.05$) either in the presence or absence of EGTA. In the control group, the elevated $[Ca^{2+}]_i$ stimulated by thrombin restored to near resting level within 1 min, while in the presence of LSPC, it took about 10 min for the increased $[Ca^{2+}]_i$ to restore to the baseline, hence a persistently high level of Ca^{2+} appeared after the stimulation both in the presence and absence of EGTA (Fig 1).

Thrombosis LSPC (600 μl·kg^{-1}) had a potent prothrombotic action on electrically stimulated arterial thrombosis (Fig 2). At 1 h, the rectal temperature was lowered approaching room temperature. This indicated that the abdominal aorta was occluded almost completely, while the thrombolytic agents showed only a trifling antithrombotic action.

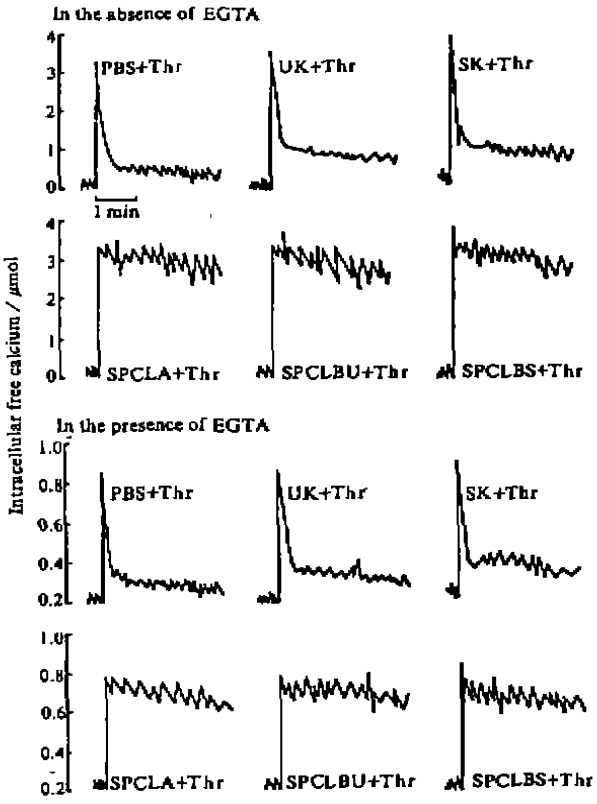

Fig 1. Actions of lytic solution of PRP clots (50 μl) and thrombolytic agents (2000 IU·ml^{-1}) on thrombin (Thr 1 IU · ml^{-1}) induced increase at the level of platelet free calcium both in the absence and presence of EGTA as indicated by fluorescence at λ_{ex}340 nm and λ_{em}510. $n=6$, all $P>0.05$ *vs* PBS.

DISCUSSION

Platelet activation occurred after thrombolytic remedies[2], and a maximum aggregation appeared at plasma concentrations of plasminogen and at blood levels of urokinase and streptokinase reached after infusing these drugs[8]. Our results, in addition to other reports[4,9], however, showed that thrombolytic agents themselves *in vitro* did not elicit potent direct potentiation on agonist-induced aggregation. Recent studies focused on the role played by plasmin[10,11] and thrombin[11,12] in

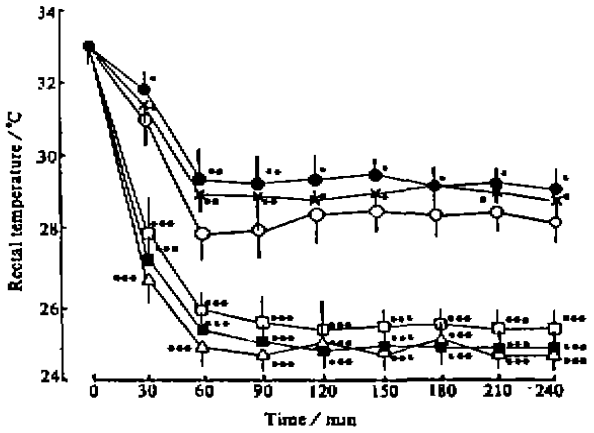

Fig 2. Actions of lytic solution of PRP clots (600 μl · kg^{-1}) and thrombolytic agents (40000 IU · kg^{-1}) on electric stimulated thrombosis of abdominal aorta in rats as presented by rectal temperature. $n=8$, $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$. Control (○); SK (●); UK (×); SPCLA (□); SPCLBS (■); SPCLBU (△).

the hyperaggregability. In terms of the molecular mechanism of platelet activation, Ca^{2+} played a pivotal role[13]. In the absence of LSPC, Ca^{2+} elevation stimulated by thrombin was transient. It indicated that Ca^{2+} homeostasis after platelet stimulation led to rapid restoration of elevated Ca^{2+} to resting level. In this study, LSPC did not show action on the influx of Ca^{2+} or the release of intracellular stored Ca^{2+}, but had an inhibitary effect on the restoration of increased cytoplasmic free Ca^{2+} induced by agonists. It is different from that of other reported aggregation modulators such as plasmin[8]. Whether this is the reason that most inhibitors of platelet aggregation showed no beneficial effects on the rethrombosis after thrombolytic therapy is to be further evaluated.

The mechanism of Ca^{2+} homeostasis after platelet stimulation has not yet been clearly elucidated. The mechanism of LSPC on Ca^{2+} homeostasis in platelet after stimulation is to be further explored.

The biochemical property of the thrombolytic products remains unclear. Fibrinogen degredation products can not be regarded as the basis underlying this phenominon, as these products were found to be capable of inhibiting platelet aggregation, adhesion, and release reaction[14]. The action of a new plasma factor[15], a protein with a small molecular weight acting synergistically to ADP-induced platelet aggregation in the thrombotic mouse plasma, in the platelet activation after thrombolytic therapy is to be elucidated.

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富含血小板的血浆凝块溶解液通过影响 Ca^{2+} 的自稳态介导的血小板高反应性

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摘要 大鼠富含血小板的血浆凝块溶解液增加血小板的聚集和丙二醛的形成，促进体内动脉血栓形成，但对凝血酶诱导的血小板钙内流及释放无影响. 然而，使诱导剂导致的血小板游离钙持续维持在高水平. 尿激酶或链激酶并不增加血小板的反应性. 因此，我们认为溶栓治疗后的血小板功能亢进是血凝块溶解产物介导的，而与溶栓药物无关.

关键词 尿激酶；链激酶；溶栓治疗；血小板聚集；钙

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Second peak of plasma diazepam concentration and enterogastric circulation

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ABSTRACT Intragastric food administration caused a pronounced second peak of plasma diazepam concentration in rabbits after iv diazepam 5 mg · kg^{-1}. The second peak disappeared after gastrostomy and choledochostomy. A large amount of diazepam was found in the gastric juice while its content in bile remained at a much lower level during the whole experiment. These results suggested that diazepam may undergo an enterogastric circulation in addition to its enterobepatic circulation, with the former mainly contributing to the appearance of the second peak.

KEY WORDS diazepam; pharmacokinetics; gastric juice; bile; gastrostomy; choledochostomy

The plasma concentration-time curve of diazepam exhibits 2 distinct peaks after a single dose[1]. The second peak appeared postprandially and seemed not to have much to do with the enterohepatic circulation[2], nor with changes in its serum protein binding[3]. In a preliminary experiment, we found that a large amount of diazepam appeared in gastric juice after iv the drug in rabbits. The present study was designed to investigate the possibility that diazepam in gastric juice should be related to the rebound of its plasma levels in rabbits.

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MATERIALS AND METHODS

Rabbits New Zealand rabbits of either sex, weighing 2.5 ± *s* 0.3 kg were used, and were fasted for 12 h before experiment.

Drugs Diazepam powder was purchased from Yiming Pharmaceutical Factory (Beijing), and diazepam injection from Shanghai № 13 Pharmaceutical Factory. Standard *N*-desmethyldiazepam was a product of Sigma Chemical Co, USA.

Drug analysis[4,5] Pipette 1 ml sample of