

Effects and mechanisms of triacetylshikimic acid on platelet adhesion to neutrophils induced by thrombin and reperfusion after focal cerebral ischemia in rats¹

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ABSTRACT

AIM: To investigate the effect of triacetylshikimic acid (TSA) on the platelet adhesion to neutrophils and P-selectin expression on activated platelet membrane induced by thrombin and reperfusion after focal cerebral ischemia. **METHODS:** The platelet adhesion to neutrophils was evaluated by rosette assay, and P-selectin expression on platelet membrane was determined by flow cytometry. **RESULTS:** TSA 10 - 1000 $\mu\text{mol/L}$ markedly inhibited thrombin(0.4 kU/L)-induced platelet adhesion to neutrophils. The platelet adhesion to neutrophils induced by a 21-h reperfusion after middle cerebral artery occlusion for 3 h was also inhibited in a dose-dependent manner by TSA 50 - 200 mg/kg given by ig immediately and at 60 min again after the onset of cerebral ischemia. TSA was also shown to decrease the P-selectin expression on platelet surface induced by thrombin in washed platelet and by adenosine diphosphate (ADP) 5 $\mu\text{mol/L}$ in whole blood. **CONCLUSION:** Reperfusion after cerebral ischemia and thrombin induced platelet adhesion to neutrophils, which could be reduced by TSA probably due to its inhibition of P-selectin expression on activated platelets.

INTRODUCTION

Interaction between platelets and leukocytes has been demonstrated to play important roles in pathophysiological

process in several animal models of vascular diseases⁽¹⁻³⁾. Platelets in the platelet-rich thrombus formed at the hemorrhagic site can recruit neutrophils which accumulate around the thrombus⁽⁴⁾. The activated platelets can also affect the ability of adhesion, aggregation, and phagocytosis of neutrophils. Platelets can bind to neutrophils or monocytes through the modulation of P-selectin (GMP-140, CD62P) expression on platelet membrane^(5,6). Previous studies suggested that platelets were activated after cerebral ischemia and reperfusion⁽⁷⁻⁹⁾. Platelet activation and the following interaction between platelets and neutrophils on the surface of cerebral microvascular endothelial cells may contribute to the development of brain injury after ischemic stroke. However, the adhesion changes of platelets to neutrophils after reperfusion following cerebral ischemia have not been observed yet. To investigate the nature of platelet adhesion to neutrophils during cerebral ischemia and reperfusion will provide alternate pathways to develop therapeutic strategies against stroke. We therefore investigated the binding of platelets to neutrophils after platelet activation by thrombin and reperfusion after middle cerebral artery occlusion in rats in this experiment.

Triacetylshikimic acid (TSA) was synthesized based on the structure of shikimic acid, which was extracted from *Fructus Anisi Stellati* (*Illicium verum* Hook f). Shikimic acid and TSA were both found to inhibit platelet aggregation induced by ADP and collagen⁽¹⁰⁾. Shikimic acid has already been demonstrated to prevent brain injury after focal cerebral ischemia induced by thrombosis⁽¹¹⁾. TSA possesses the advantage over shikimic acid of being a lipid soluble compound and readily being absorbed from gastrointestinal tract. Therefore, the ability of TSA to prevent ischemic injury and platelet adhesion to neutrophils after platelet activation induced by thrombin and reperfusion after cerebral ischemia were also evaluated in the study.

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MATERIALS AND METHODS

Agents TSA (purity 98.39 %), synthesized by Department of Phytochemistry, Beijing University of Chinese Medicine, was made into emulsion with 2 % Tween 80 for *in vivo* use. *In vitro* experiment, TSA was dissolved with ethanol and diluted with phosphate buffer (in mmol/L: KH_2PO_4 10, Na_2HPO_4 37, NaCl 87, and KCl 53) yielding the final concentration of 0.02 % ethanol. Dextran (T 500, Pharmacia); Goat polyclonal antibody against P-selectin (Santa Cruz Biotechnology Inc, USA); FITC labeled rabbit anti-goat IgG (1:10, Biotinge Biomedicine Co, USA).

Platelet-neutrophil adhesion The adhesion of platelets to neutrophils was determined by rosette assay. Blood was extracted from common carotid artery of rats and anticoagulated with 2.7 % edetic acid. Platelet-rich plasma, which was obtained by centrifugating the blood at $120 \times g$ for 10 min, was then centrifuged at $1200 \times g$ for 10 min to obtain platelet pellet. The pellet was washed by PBS containing 1 % bovine serum albumin and edetic acid 1.4 mmol/L for three times and the density of platelets was adjusted to $1 \times 10^{11}/\text{L}$.

Neutrophils were isolated from the same blood sample. After the platelet-rich plasma was removed, the blood volume was reconstituted with saline. Then one to ten volumes of 6 % dextran was added and the blood was incubated at 37 °C for 40 min without stirring. The upper layer of the blood was collected and added on top of 3-mL lymphocyte separation medium, centrifuged at $600 \times g$ for 45 min. The neutrophil pellet was washed with PBS. The contaminated erythrocytes were removed by hypotonic method, washed with PBS for three times, and then resuspended in Hanks' solution with the concentration of neutrophils adjusted to $4 \times 10^9/\text{L}$.

Rosette assay was performed according to the method described by Hamburger and McEver^[5] with some modifications. Briefly, 10 μL of thrombin solution (1, 2, 3, 4, or 6 kU/L) was added into 100 μL of platelet suspension yielding a final concentration of 0.1 – 0.6 kU/L. After reaction for 10 min at 20 °C without any stirring, same volume of 2 % paraformaldehyde was added for platelets to be fixed for 30 min. Then, 100 μL of neutrophil suspension was added into the fixed platelet suspension and incubated under agitation at 4 °C for 30 min. Each of platelet-neutrophil suspension was placed on a microscope slide over which a coverslip were positioned. Three hundred neutrophils were scored for the presence of platelets. Neutrophils bearing two or

more platelets were defined as rosettes. When the effect of TSA was evaluated, the solution of TSA and aspirin (ASP) were added 30 min before platelet activation.

Cerebral ischemia and reperfusion model

Male Wistar rats, weighing (224 ± 21) g, (Grade II, Certificate No 01-3008), supplied by the Experimental Animal Center, Chinese Academy of Medical Sciences, were anesthetized with 10 % chloral hydrate (350 mg/kg, ip). Room temperature was maintained at 24 °C – 25 °C. Focal cerebral ischemia was performed by middle cerebral artery occlusion (MCAO) according to the method described previously^[12]. Briefly, the right common carotid artery, external (ECA), and internal carotid artery (ICA) were exposed through a ventral middle incision in the neck. A monofilament suture (ϕ 0.26 mm) was inserted into ICA from ECA and put forward about 20 mm from the origin of ICA into the intracranial segment of ICA to block the origin of middle cerebral artery. Three hours after the onset of the occlusion, the suture was withdrawn and recirculated for 21 h.

In vivo experimental protocols The rats were divided into 5 groups: sham group; vehicle group, subjected to 21-h reperfusion after 3-h MCAO and given ig 2 % Tween 80 2 mL/kg; TSA 50, 100, and 200 mg/kg group subjected to 21-h reperfusion after 3-h MCAO and given TSA ig immediately and at 60 min again after the onset of ischemia. Twenty-one hours after reperfusion, the neurological deficit score of each rat was evaluated. The total score of ten was evaluated according to the modified methods of Bederson *et al*^[13] and Lin *et al*^[14]. The test was performed as following: ① Rats were gently suspended by the tail, the left forelimb was flexed, scored 1 – 3 according to the severity. When the rat was circling to the right side during suspension, scored 4. ② Rats were allowed to grasp fine metal net and pulled gently backward by the tail, the left forelimb showed decreased strength and reduced ability to hold the net compared with the right limb and scored 1 – 3. ③ Rats were placed on a smooth plane, the lateral push resistance toward the left side decreased and scored 1 – 3.

For evaluation of platelet adhesion to neutrophils after reperfusion after focal cerebral ischemia, blood was withdrawn from common carotid artery after 21-h reperfusion and the rosette assay was carried out as described above.

P-selectin expression on activated platelet membrane Blood was obtained from rat common carotid artery and was anticoagulated with ACD (in mmol/L: citric acid 71, trisodium citrate 85, dextrose

110, ACD: blood = 1:6). Platelets were suspended in modified Tyrode's buffer (in mmol/L: NaCl 137, KCl 2.8, MgCl₂ 1, NaHCO₃ 12, Na₂HPO₄ 0.4, BSA 0.35 %, HEPES 10, and glucose 5.5, pH 7.4) yielding a platelet concentration of 2×10^{11} /L. Platelet suspension solution 5 mL was aliquoted into polyethylene tube containing 50 μ L of Tyrode's buffer and 5 μ L of drug solution (at a final concentration of TSA 1, 10, and 100 μ mol/L or ASP 10 μ mol/L). Then the platelet suspension was incubated with thrombin 0.4 kU/L and goat polyclonal antibody against P-selectin 200 mg/L for 15 min without stirring. Platelets were fixed with 1 % paraformaldehyde at room temperature for 30 min and then incubated with 5 μ L of FITC-labeled rabbit anti-goat IgG (1:10) for 15 min. The sample was diluted with 500 μ L of Tyrode's buffer and analyzed in a EPICS XL cytometer (Coulter, USA). The fluorescent intensity of platelets and percent of positive labeled platelets were recorded.

P-selectin expression on platelet membrane in whole blood was also studied. The procedure of the assay was the same as described above except for whole blood being used instead of platelet suspension. Platelets were activated by ADP 5 μ mol/L.

Statistical analysis The data were expressed as $\bar{x} \pm s$ and compared by *t*-test.

RESULTS

Thrombin induced platelet-neutrophil adhesion Only 11 % of neutrophils bound two or more platelets prior to activation by thrombin. In contrast, the percent of neutrophils bearing two or more stimulated platelets increased by 1.5, 1.8, 2.1, 2.8, and 2.7 times respectively after application of thrombin (0.1, 0.2, 0.3, 0.4, and 0.6 kU/L) in platelet suspension (Tab 1).

Tab 1. Platelet adhesion to neutrophils induced by thrombin. Values are percent of rosette formation. $n = 6$. $\bar{x} \pm s$. * $P < 0.01$ vs normal (without thrombin) group.

Thrombin/kU·L ⁻¹	Neutrophil binding platelets/%
0 (Normal)	11.0 ± 2.7
0.1	26.5 ± 2.8 ^c
0.2	30.5 ± 2.8 ^c
0.3	33.8 ± 2.9 ^c
0.4	42 ± 4 ^c
0.6	42 ± 4 ^c

When TSA was employed directly to platelet suspension 30 min prior to the application of thrombin and yielded the final concentrations of 10, 30, 100, and 1000 μ mol/L, the rosette formation induced by thrombin 0.4 kU/L was reduced by 11 % ($P < 0.05$), 24 % ($P < 0.01$), 32 % ($P < 0.01$), and 34 % ($P < 0.01$), respectively. However, TSA 1 μ mol/L did not inhibit the rosette formation induced by thrombin. ASP inhibited the rosette formation only at the highest concentration (2800 μ mol/L) used in this experiment (Tab 2).

Tab 2. Effect of triacetylshikimic acid (TSA) and aspirin (ASP) on platelet-neutrophil adhesion induced by thrombin 0.4 kU/L. TSA or ASP was applied directly to platelet suspension 30 min prior to activation by thrombin. Values are percent of rosette formation. $n = 5$. $\bar{x} \pm s$. * $P < 0.01$ vs normal group. ^a $P < 0.05$, ^b $P < 0.01$ vs vehicle group.

Groups	Neutrophil binding platelets/%
Normal	11 ± 3
Vehicle	43 ± 3 ^c
TSA 1 (μmol/L)	41.6 ± 2.7
TSA 10 (μmol/L)	38.2 ± 2.8 ^c
TSA 30 (μmol/L)	33 ± 4 ^f
TSA 100 (μmol/L)	29.0 ± 1.6 ^f
TSA 1000 (μmol/L)	28.4 ± 2.1 ^f
ASP 28 (μmol/L)	40.8 ± 2.8
ASP 280 (μmol/L)	40.0 ± 2.5
ASP 2800 (μmol/L)	37.2 ± 2.9 ^c

Platelet-neutrophil adhesion after cerebral ischemia and reperfusion The percent of neutrophils rosetted two or more platelets after 21-h reperfusion following 3-h focal cerebral ischemia was significantly increased from 16.6 % ± 2.8 % of sham group to 39 % ± 5 % ($P < 0.01$) of vehicle group. TSA, given immediately and at 60 min again after the onset of cerebral ischemia, markedly decreased the number of platelet-neutrophil rosettes in a dose-dependent manner. The percentages of rosettes were decreased by 12 % ($P > 0.05$), 29 % ($P < 0.01$), and 30 % ($P < 0.01$) by TSA treatment at the dose of 50, 100, and 200 mg/kg, respectively. The neurological deficit scores after cerebral ischemia/reperfusion were also reduced significantly by TSA at the dose of 100 and 200 mg/kg ($P < 0.01$, Tab 3).

Tab 3. Effect of triacetylshikimic acid (TSA) on cerebral ischemia/reperfusion-induced platelet-neutrophil adhesions. Platelet adhesions to neutrophils were evaluated by rosette formation, and neurological deficit scores were determined before drawing the blood. $\bar{x} \pm s$. $^cP < 0.01$ vs sham group. $^eP < 0.05$, $^fP < 0.01$ vs vehicle group.

Groups	n	Neutrophil binding platelets/ %	Neurological deficit scores
Sham	8	16.6 ± 2.8	0 ± 0
Vehicle	9	39 ± 5 ^c	5.7 ± 1.4
TSA 50 (mg/kg)	8	34 ± 5	4.8 ± 0.7
100	9	28 ± 7 ^f	4.3 ± 1.0 ^e
200	8	24 ± 7 ^f	4.2 ± 0.9 ^e

P-selectin expression on platelet membrane

The fluorescence intensity (FI) and the percent of labeled platelets stimulated by thrombin 0.4 kU/L in platelet suspension were significantly increased from 5.4 % ± 1.6 % and 4.81 % ± 0.23 % of the normal untreated group to 7.4 % ± 0.7 % and 11 % ± 3 % of vehicle group, respectively ($P < 0.05$). However, the percentages of fluorescence labeled platelets were reduced by 28 % ($P > 0.05$), 38 % ($P < 0.05$), and 45 % ($P < 0.05$) after application of TSA 1, 10, and 100 μmol/L, respectively. ASP 10 μmol/L did not decrease the fluorescence intensity and percent of labeled platelets after platelet activation by thrombin (Tab 4).

ADP 5 μmol/L also induced significant increases in the fluorescence intensity and percent of labeled platelets in whole blood preparation ($P < 0.05$), which were reduced by TSA 100 μmol/L significantly ($P < 0.01$). TSA 1, 10 μmol/L and ASP 10 μmol/L also reduced the percent of positive platelets by 17 % ($P > 0.05$), 24 % ($P > 0.05$), and 11 % ($P > 0.05$), respectively (Tab 4).

DISCUSSION

Platelet-neutrophil adhesion has been studied in patients with unstable angina and coronary angioplasty^[15,16]. We investigated the platelet-neutrophil adhesion changes after reperfusion following focal cerebral ischemia in rats in the study. The results indicate that reperfusion after cerebral ischemia can induce platelet adhesion to neutrophils that may be related to platelet activation after the insult. Platelets may mediate the interaction between neutrophils and vascular endothelial cells and promote the formation of thrombus in microcirculation^[17]. The interaction between neutrophils and microvascular endothelial cells induced by activated platelets may lead to the infiltration of neutrophils into the brain which is implicated in the development of reperfusion-induced brain injury after cerebral ischemia^[18,19]. Therefore, platelet and neutrophil interaction may also play an important role in the development of reperfusion-induced brain injury after cerebral ischemia.

To further address the nature of platelet adhesion to neutrophils, the adhesion of activated platelets to neutrophils *in vitro* was investigated. The results, which indicated that the adhesion of platelet to neutrophils was significantly enhanced after platelet activation by thrombin, were consistent with previous reports^[5,20]. The platelet-neutrophil adhesion may be mediated through the expression of P-selectin on activated platelet surface^[21]. Our study demonstrated that thrombin- and ADP-induced increases in fluorescence intensity and percent of platelet labeled by P-selectin antibody suggested that P-selectin expression on platelet membrane increased after platelet activation. The adhesion of activated platelet to neutrophils may be due to the P-selectin expression on

Tab 4. Effect of triacetylshikimic acid (TSA) and aspirin (ASP) on P-selectin expression induced by thrombin on separated platelets and induced by ADP (5 μmol/L) on platelets in whole blood. Fluorescence intensity (FI) and the percent of positive cells bound FITC-monoclonal antibody against P-selectin were determined by flow cytometry. n = 4. $\bar{x} \pm s$. $^bP < 0.05$ vs normal group. $^cP < 0.05$ vs vehicle group.

Groups	Thrombin-induced platelet suspension		ADP-induced platelet in whole blood	
	FI	Positive platelets/ %	FI	Positive platelets/ %
Normal	5.4 ± 1.6	4.81 ± 0.23	3.2 ± 0.9	4.8 ± 0.3
Vehicle	7.4 ± 0.7 ^b	11 ± 3 ^b	4.9 ± 1.2 ^b	10 ± 3 ^b
TSA (μmol/L) 1	6.5 ± 0.6	8 ± 3	3.92 ± 0.12	8 ± 4
10	6.2 ± 0.7 ^e	6.6 ± 2.5 ^e	3.7 ± 0.3	7.3 ± 2.4
100	5.9 ± 0.9 ^e	5.8 ± 1.1 ^e	3.5 ± 0.3 ^e	5.9 ± 1.7 ^e
ASP (μmol/L) 10	6.4 ± 0.9	9 ± 3	4.04 ± 0.12	9 ± 3

platelet membrane. Further investigation should be performed to address function of P-selectin on platelet and neutrophil adhesion induced by reperfusion after cerebral ischemia.

Triacetylshikimic acid (TSA) inhibits thrombin and cerebral ischemia/reperfusion-induced platelet-neutrophil adhesion, which may be related to its inhibitory effect on P-selectin expression on platelet surface. TSA has recently been shown to reduce reperfusion-induced brain injury after focal cerebral ischemia^[22]. The effect of TSA on platelet-neutrophil interaction may contribute to its protective effect on brain ischemia. The results of this study suggest that TSA is deserved to be developed as a therapeutic candidate for ischemic stroke in the further work.

In conclusion, thrombin and reperfusion after focal cerebral ischemia induced platelet adhesion to neutrophils, which could be inhibited by TSA. Effects of TSA on P-selectin expression on platelet membrane might contribute to its preventive effect on the adhesion.

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三乙酰莽草酸对凝血酶和局灶性脑缺血再灌注诱导的血小板与白细胞粘附的作用及其机制¹

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关键词 三乙酰莽草酸; 脑缺血; 血小板; 中性白细胞; P-选择素

目的: 研究三乙酰莽草酸(TSA)对凝血酶和局灶性脑缺血再灌注诱导的血小板与中性粒细胞粘附及活化血小板膜 P-选择素表达的作用。 **方法:** 观察玫瑰花环形成率作为血小板与中性粒细胞粘附率指标,

并用流式细胞仪测定血小板表面 P-选择素的表达。 **结果:** TSA 10-1000 $\mu\text{mol/L}$ 可明显抑制凝血酶 0.4 kU/L 诱导的血小板与中性粒细胞的粘附。 TSA 50-200 mg/kg 剂量依赖性抑制大鼠中动脉阻断 3 h 再灌注 21 h 引起的血小板与中性粒细胞的粘附。 TSA 可明显抑制凝血酶诱导的血小板 P-选择素的表达和 ADP 5 $\mu\text{mol/L}$ 诱导的全血中血小板膜表面 P-选择素的表达。 **结论:** 脑缺血再灌注和凝血酶引起血小板和中性粒细胞粘附, TSA 对 P-选择素表达的影响可能是其抑制血小板与中性粒细胞粘附的重要机制。

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