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Regular Article

Salvianolic acid B inhibits platelets as a $P2Y_{12}$ antagonist and PDE inhibitor: Evidence from clinic to laboratory

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ABSTRACT

Salviae miltiorrhiza (Danshen) has been used for thousands of years in China and some other Asian countries to treat atherothrombotic diseases. Salvianolate which consists of three water-soluble ingredients purified from Salviae miltiorrhiza, has been approved by Chinese SFDA to treat coronary artery disease. So far, there is no evidence clearly showing the clinical efficiency of salvianolate and the underlying mechanism. This study is to evaluate the effects of salvianolate on platelets in patients with acute coronary syndrome and explore the underlying mechanism. We evaluated the effects of salvianolate on platelets in patients with acute coronary syndrome by measuring ADP-induced PAC-1 binding and P-selectin expression on platelets. Salvianolate significantly potentiated the antiplatelet effects of standard dual antiplatelet therapy. We also investigated the antiplatelet effects of salvianolatic acid B (Sal-B), the major component which composes 85% of salvianolate. Sal-B inhibits human platelet activation induced by multiple agonists *in vitro* by inhibiting phosphodiesterase (PDE) and antagonizing $P2Y_{12}$ receptor. For the first time, we show the antiplatelet efficiency of salvianolate in ACS patients undergoing treatment with clopidogrel plus aspirin, and demonstrate that Sal-B, the major component of salvianolate inhibits human platelet activation via PDE inhibition and $P2Y_{12}$ antagonism which may account for the clinical antiplatelet effects of salvianolate. Our results suggest that Sal-B may substitute salvianolate for clinical use.

Introduction

Arterial thrombotic diseases, such as heart attack and stroke, are the leading cause of morbidity and mortality worldwide. Platelet activation triggered by atherosclerotic plaque disruption or endothelium injury caused by percutaneous coronary intervention (PCI) and the consequent intravascular arterial thrombogenesis is the common pathological basis of heart attack and stroke; therefore, antiplatelet drugs are effective for prevention and treatment of coronary artery disease (CAD) and stroke.

Currently, cyclooxygenase inhibitor aspirin, thienopyridine $P2Y_{12}$ receptor antagonists clopidogrel and prasugrel, fibrinogen receptor antagonists, and phosphodiesterase (PDE) inhibitor cilostazol, are the mainly used antiplatelet drugs. Among these antiplatelet drugs for arterial thrombotic diseases, aspirin and $P2Y_{12}$ receptor antagonists are most successfully and most widely used for coronary heart disease and stroke [1], while the PDE inhibitor cilostazol is used mainly in peripheral arterial occlusion and is under clinical trials in patients undergoing PCI in combination with clopidogrel and aspirin [2–5].

Though the current antiplatelet drugs are proven to be beneficial to patients with coronary heart disease, stroke and peripheral arterial disease, morbidity and mortality are still high, and novel antiplatelet agents with improved efficacy and safety are still needed. Traditional Chinese medicine (TCM) Salviae miltiorrhiza (Dangshen) has been used clinically for the treatment of cardiovascular diseases in China and some other Asian countries for thousands of years. Salvianolate, also known as depsides salts, the active components extracted from Salviae miltiorrhiza, was approved by Chinese SFDA in 2005 and widely used in clinical practice to treat CAD. However, there are few evidences

Abbreviations: Sal-B, salvianolatic acid B; VASP, vasodilator-stimulated phosphoprotein; AFM, atomic force microscopy; PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; cAMP, 3,5-cyclic adenosine monophosphate; aspirin, acetylsalicylic acid; PRP, platelet-rich plasma; HPLC, high-performance liquid chromatography.

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clearly showing its clinical efficiency as most clinically used TCMs, and the underlying mechanism is also not clear.

Salvianolate is the mixture of salvianolic acid B (Sal-B, ≥ 85%), salvianolic acid A (Sal-A, ≥ 1.9%) and rosmarinic acid (RA, ≥ 10.1%) [6]. Huang et al have found that Sal-A inhibits human platelet activation in vitro via PI3K inhibition [7]. As the most abundant ingredient of salvianolate, Sal-B has also been reported to inhibit rat platelet activation by targeting integrin α2β1 [8]. Given the pivotal roles of platelets in CAD and the proven benefits of antiplatelet drugs in treating CAD, we hypothesize that salvianolate may exert its clinical effects via platelet inhibition in CAD patients. In this study, we evaluated the effects of salvianolate on platelets in acute coronary syndrome (ACS) patients and found that it potentiates the antiplatelet effects of standard dual antiplatelet therapy. We also investigated the antiplatelet effects of Sal-B, the major component of salvianolate and demonstrated that Sal-B inhibited platelet activation induced by multiple agonists by inhibiting PDE and antagonizing $P2Y_{12}$ receptor.

Methods

Study Design to Evaluate the Antiplatelet Effects of Salvianolate in ACS Patients

Sixty three consecutive patients (Supplemental Table 1) were included after obtaining informed consent in abidance with the Declaration of Helsinki. Patients between 18 - 75 years old with chest pain suggestive of new-onset ACS were eligible, according to the 2007 ACC/AHA guideline [9]. All patients enrolled were admitted to the Department of Cardiology in Huashan Hospital, Fudan University. Exclusion criteria were previous diagnosis of ACS before and relapse, long-term use of antiplatelet or anticoagulant therapy, receiving or scheduled use of platelet GP IIb/IIIa antagonist, and history of coagulopathy.

Patients were randomly divided into two groups (Supplemental Table 1): the salvianolate group was given salvianolate (Shanghai Green Valley Pharmaceutical Co., Ltd, Shanghai, China) 200 mg/day intravenously in addition to standard treatment (according to the practice guideline of ACC/AHA) [9], and the control group was given standard treatment only (Fig. 1A). Salvianolate drip ended after one week. Current guidelines were followed for patient management and therapy, including percutaneous coronary interventions and thrombolysis. Drug therapy includes aspirin (Bay-aspirin) and clopidogrel (Plavix) (the loading dose of 300 mg, followed by a regimen therapy of 100 mg/d and 75 mg/d, respectively), statins (Lipitor 20 mg/d), LMWH (4000 IU twice daily, injected subcutaneously for 3 days) (Fig. 1A). To evaluate platelet activation status, PAC-1 binding and CD62P expression on platelet surface were determined by flow cytometry (FACS) using blood collected before and after antiplatelet treatment 7 days later, according to the protocol shown in Fig. 1A.

PAC-1 Binding and CD62P Expression Assay in Whole Blood from ACS Patients

Whole blood FACS analysis of PAC-1 binding and CD62P expression on platelet surface was taken before and 7 days after antiplatelet treatment. PAC-1 binding and CD62P expression were determined on resting and ADP-activated platelets from ACS patients, using monoclonal antibodies PAC-1 and anti-CD62P for activated αIIbβ3 and P-selectin, respectively, as reported before [10].

Blood was collected from an antecubital vein into Venous Blood Collection tubes containing 0.5 mL buffered sodium citrate (equiv. To 3.2% sodium citrate, Franklin Lakes, NJ, USA). The first 2 mL of blood were discarded. Isotonic HEPES Tyrode buffer was used to dilute the blood. Resting and platelets activated by ADP (10 μM) for 3 min were incubated with FITC-conjugated PAC-1 (Santa Cruz, San Diego, CA, USA) and PE-conjugated anti-CD62P (AbD Serotec, Oxford, UK) antibodies in the dark at room temperature for 15 min without stirring. PAC-1 binding and CD62P expression were subsequently measured using a FACS (FACSCalibur, Becton Dickinson). Results are expressed as percent of events designated positive for the marker of interest [10].

Preparation of Platelet Rich Plasma (PRP) and Washed Platelets from Normal Humans

All experiments using human samples were performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board, Fudan University. The healthy volunteers (staff or students at Fudan University) without taking aspirin or other non-steroidal anti-inflammatory drugs for at least two weeks were recruited after the informed consent was obtained. Blood (36 ml) was drawn into tubes containing 6 ml ACD (85 mM sodium citrate, 71.38 mM citric acid, and 27.78 mM glucose) solution. PRP and washed platelets were prepared as reported before [11–13].

Platelet Aggregation Assay

Aggregation of 0.5 ml human washed platelets in response to agonists or antagonists was analyzed using a lumi-aggregometer (Model 400VS; Chrono-Log, Havertown, PA, USA) under stirring condition (900 rpm) at 37 °C as reported before [11–14]. Platelet aggregation was initiated by addition of agonists with or without preincubating platelets with Sal-B (HPLC > 99%, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China) for 3 min. Each sample was allowed to aggregate for at least 3 min. The baseline was set using Tyrode's buffer as blank.

Assay of PAC-1 Binding on Platelets from Normal Humans

Whole blood from normal humans was incubated with Sal-B at different concentrations. PAC-1 binding on the surface of resting or ADP activated platelets was determined by FACS as described above.

$[Ca^{2+}]_i$ Measurements

Human PRP was prepared as described above. 1 mg Fluo-3/AM (Biotium, Hayward, CA, USA) was dissolved in DMSO with 20% F-127 as 1 mM stock solution [15]. Platelets pelleted from PRP were resuspended in Ca^{2+}-free Tyrode's solution, and then incubated with 2 μM Fluo-3/AM for 30 min at 37 °C. After washing twice, the fluo-3-loaded platelets were finally suspended in Tyrode's buffer containing 0.5 mM EGTA, at a density of 3×10^8 platelets/ml. The fluo-3-loaded platelets were preincubated with Sal-B at 37 °C for 3 min before the addition of the platelet agonists. Fluorescence (excitation 505 nm, emission 530 nm) was measured with a fluorescence spectrophotometer (Model F4500; Hitachi, Tokyo, Japan) [15].

Western Blotting Analysis of VASP (Vasodilator-Stimulated Phosphoprotein) Phosphorylation in Normal Human Platelets

Washed platelets was prepared as described above and treated or untreated with 5 μM prostaglandin E1 (PGE1) at 37 °C for 30 min [16], followed by incubation with Sal-B (70, 140, 280 μM) or AR-C69931MX (100 nM, a gift from AstraZeneca, Loughborough, UK) under stirring condition (900 rpm) for 3 min. After stimulation with 10 μM ADP for 3 min the reaction was stopped by addition of 5 x sample buffer and boiled for 5 min. Platelet lysates were further subjected to western blot analysis of VASP phosphorylation using anti-pSer157-VASP (Cell signalling, Beverly, MA, USA)[17,18]. The optical density of the bands was measured using Image J (National Institutes of Health, Bethesda, MD).