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Neferine exerts its antithrombotic effect by inhibiting platelet aggregation and promoting dissociation of platelet aggregates

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ABSTRACT

Introduction: Neferine, a kind of isoquinoline alkaloid, extracted from the seed embryo of *Nelumbo nucifera Gaertn*, has long been recognized in traditional medicine as a medicinal plant with various usages. Neferine has many biological activities, including anti-hypertensive, anti-arrhythmic, negative inotropic effect and relaxation on vascular smooth muscle. Although previous studies have reported its antithrombotic effect, the mechanisms by which it exerts antithrombotic effect have not been thoroughly studied.
Method: Washed mice platelets and mice platelet-rich-plasma (PRP) were used to investigate the effects of neferine on platelet aggregation, secretion induced by various agonists and dissociation of agonist-formed platelet aggregates. Bioflux plates coated with collagen were used to investigate the effect of neferine on platelet adhesion and thrombosis in vitro. With collagen-epinephrine-induced acute pulmonary thrombus formation mouse model, the effect of neferine on thrombosis in vivo was also examined.
Results: Neferine, significantly and dose-dependently, inhibited collagen-, thrombin-, U46619-induced platelet aggregation in mice washed platelets, or ADP-induced platelet aggregation in PRP. Neferine treatment decreased platelet dense granule secretion initiated by collagen, thrombin and U46619. Also, Neferine dramatically and dose-dependently promoted the dissociation of platelet aggregates pre-formed by various agonists including collagen, thrombin, U46619 or ADP. Neferine can significantly reduce the area of mice platelets adhesion to the collagen and inhibit thrombosis in vitro. In collagen-epinephrine-induced acute pulmonary thrombus mouse model, neferine, at 6 mg/kg, significantly attenuated thrombus formation.
Conclusions: Neferine remarkably prevents thrombus formation by inhibiting platelet activation, adhesion and aggregation, as well as promoting disassembly of pre-formed platelet aggregates. The inhibitory effects of neferine on platelet activation might be relevant in cases involving aberrant platelet activation where neferine could be used as an anti-platelet and antithrombotic agent.

Introduction

Platelets, the tiniest anucleated cell fragments in peripheral blood stream with an average lifespan of 5–9 days, are derived from the fragmentation of precursor megakaryocytes. Platelets play critical roles in the maintenance of vascular integrity by adhering to the damaged endothelium, and forming platelet thrombi. Although platelet activation and subsequent thrombus formation at sites of vascular injury are crucial for normal hemostasis, they also play fundamental roles in atherogenesis, myocardial infarction and stroke [1–6].

When platelets are exposed to chemical stimuli such as ADP, thrombin, and collagen, they transform into an adhesive protruded state from a smooth, non-adhesive state, followed by release and expression of biologically active substances, especially activation of integrin αIIbβ3-the major integrin on the platelet surface which endows platelets with the ability of binding plasma fibrinogen. At damaged sites of vessels, accumulation of thrombin, exposed collagen and von Willebrand factor (vWF) quickly activate platelets, which adhere to and spread on injured vessel walls to form an initial monolayer of platelets. Although the monolayer of activated platelets could not completely block bleeding, amplification of thrombin generation by activated platelets as well as the bioactive molecules derived from the activated platelets, including ADP and thromboxane A2(TxA2), stimulate and recruit more circulating platelets to the top of the initial monolayer of platelets, forming stable platelet plug bridged mainly by

Abbreviations: Nef, Neferine; PRP, platelet-rich plasma; PPP, platelet-poor plasma; U46619, 9,11-Dideoxy -9a,11a -methanoepoxy prostaglandin F2a; TxA2, thromboxane A2; ADP, adenosine diphosphate; ATP, adenosine triphosphate; vWF, von Willebrand factor; DMSO, dimethyl sulfoxide; PGE1, Prostaglandin E1; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography.

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the interaction of activated integrin αIIbβ3 on the surface of platelets with its ligands including fibrinogen and vWF.

Besides the capability to aggregate in response to various stimuli, platelets also possess intrinsic abilities to disaggregate. In the past, few studies focused on the mechanism of platelet disaggregation, however, it has been shown that the dysregulation of platelet disaggregation also causes bleeding or thrombotic problems [7].

In the past few decades, people have been committed to find drugs to inhibit pathological aggregation of platelets from natural sources. Neferine, an isoquinoline alkaloid extracted from the seed embryo of *Nelumbo nucifera Gaertn*, has been demonstrated to have a wide range of biological activities, such as antihypertensive, antiarrhythmic, antiagglutinating, antioxidant, negative inotropic effect and relaxation on vascular smooth muscle, etc. [8–10]. Previous studies have shown that neferine is able to inhibit platelet aggregation in PRP. In the current study, we investigated the antithrombotic effects of neferine with washed mice platelets, PRP, as well as in vitro and in vivo thrombus formation mouse model. We found that neferine remarkably prevents thrombus formation by inhibiting platelet activation and aggregation, as well as promoting disassembly of pre-formed platelet aggregates.

Material and Methods

Reagents and Animals

Neferine (≥97% purity, measured by HPLC) (Fig. 1) was provided by the Department of Pharmacology's Phytochemistry Laboratory, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China)[10]. Stock solution of neferine (1 mM) was prepared by dissolving neferine in hydrochloric acid (HCL), then was diluted with Tyrode's buffer to a concentration range of 0.3 μM to 3 μM for use. The stock solution was stable for at least 1 month at 4 °C. Thrombin, U46619, Aspirin and ADP were obtained from Sigma (St. Louis, MO, USA). Collagen and Chrono-Lume Kit were purchased from Chrono-log (Haver-town, PA, USA). Epinephrine was purchased from Yuanda(Wuhan, China). Cell- Trace™ Calcein Green from Invitrogen (Carlsbad CA, USA). DMSO were purchased from Amresco (Solon, OH, USA). All other chemicals were of analytical grade.

Male Kunming mice weighing 18–22 g were provided by the Experimental Animal Center of Tongji Medical College. All animals were maintained in a standard laboratory animal facility with free access to feed, water and acclimated for at least 2 weeks before use. The study protocol was reviewed and approved by the ethics committee (Ethics Committee for experimental animals, Tongji Medical college, No.13, Hangkong Road, Wuhan, P.R. China) and in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996).

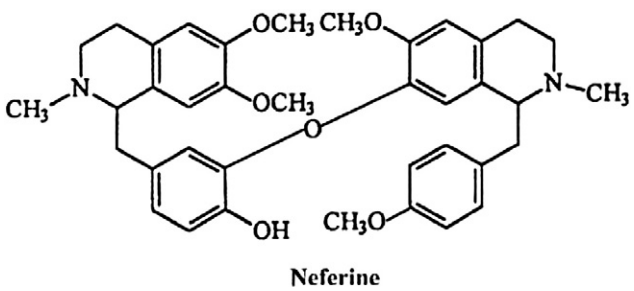

Fig. 1. The molecular structure of neferine.

Platelet and washed red blood cell preparation and aggregation

Mouse blood was drawn from the abdominal vein without stasis into the siliconized vacuum containers containing 1: 9 (v/v) 3.8% sodium citrate. Platelet-rich plasma (PRP) was obtained by spinning whole anticoagulant blood for 10 min at 150 ×*g*. Platelets were pelleted by centrifuging PRP at 800 ×*g* for 10 min. The supernatant was allocated to a platelet-poor plasma (PPP) fraction, which was used as a reference solution in aggregation assays. Platelets were washed twice with Tyrode's buffer (137 mM NaCl, 13.8 mM $NaHCO_3$, 5.5 mM glucose, 2.5 mM KCl, 20 mM HEPES, 0.36 mM NaH_2PO_4, pH 7.4) containing 1 μM PGE1 and 2.5 mM EDTA, then were resuspended in Tyrode's buffer. $CaCl_2$ (1 mM) was added prior to the agonist stimulation. All platelet preparations were conducted at room temperature.

To prepare washed red blood cells, the remaining blood after PRP collection, consisting mainly of red blood cells, was washed with Tyrode's buffer and centrifuged three times at 800 ×*g* for 10 min to remove plasma proteins.

Platelet aggregation was performed as previous described [11]. Briefly, washed mouse platelets (0.25 ml; 3.00×10^8 mL^{-1}) were preincubated with neferine (0.3-3 μM) for 5 min at 37 °C without stirring. The agonists were then added with stirring at 1000 rpm to induce platelet aggregation. The data is represented either in the form of actual aggregation tracings or maximum aggregation percentage calculated by taking agonist-induced aggregation of untreated platelets as 100%.

Platelet disaggregation

The effect of neferine on the disaggregation of platelet aggregates formed by various agonists in PRP or washed platelet suspension was measured by adding neferine into test tube when the maximal aggregation was achieved. The percent platelet disaggregation (%PD) was characterized by an %PD determined as a ratio %PD = [(T1 − T2)/T1] × 100%, where T1 is the maximal value of light transmission of the platelet suspension on aggregation and T2 is the minimal value of light transmission of the platelet suspension on disaggregation determined for 10 min[12]. Percent platelet disaggregation (%PD) was normalized by subtracting respective vehicle control %PD for each experiment.

Platelet dense granule secretion measurement / ATP release assay

The inhibitory effect of neferine on collagen-, thrombin- and U46619-induced platelet dense granule secretion was investigated with washed mice platelets by measuring the release of adenosine triphosphate (ATP) from platelets with Chrono-Lume (Chrono-log) [13,14]. Briefly, platelets were preincubated with neferine or Tyrode's buffer for 5 min at 37 °C, then activated by collagen in a lumi-aggregometer at 37 °C with stirring at 1000 rpm. The data is represented either in the form of actual secretion tracing or normalized maximal extent of secretion taking collagen stimulated sample as 100%.

Thrombus on immobilized collagen under flow conditions

Bioflux plates (Bioflux 200 from Fluxion, California, USA) were coated with 80 μg/ml collagen, and incubated over night at 4 °C. Washed mice platelets were preincubated with Tyrode's buffer, aspirin (100 mM) and neferine (0.3 μM, 3 μM) for 5 min. Then Cell Trace™ Calcein Green(0.34 μM, 30 min at 37 °C) was used to label mice washed platelets. Labeled platelets were then mixed with washed red blood cells and $CaCl_2/MgCl_2$ (75 mM/37.5 mM) mixture [15], and perfused through Bioflux plates for 5 min at a wall shear rate of 1000 per seconds. Platelet adhesion was visualized with a 40× long working distance objective in real time for both fluorescent and transmitted light microscopy. Image sequences of the time lapse were recorded using the Nikon Element D