Thrombosis Research 133 (2014) 211–217

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside (THSG) attenuates human platelet aggregation, secretion and spreading in vitro

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ARTICLE INFO

Article history:
Received 27 August 2013
Received in revised form 7 October 2013
Accepted 7 November 2013
Available online 15 November 2013

Keywords:
Platelet
THSG
Aggregation
Granule release
Fc γ RIIa

ABSTRACT

Introduction: 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside(THSG) is a water-soluble component of the rhizome extract from the traditional Chinese herb *Polygonum multiflorum*. Recent studies have demonstrated that THSG has potent anti-oxidative and anti-inflammatory effects. In this study, we investigated the anti-platelet aggregation, secretion and spreading of THSG with different methods. The purpose was to explore the anti-platelet effect of THSG and the underlying mechanism.
Materials and Methods: We investigated the anti-platelet activity of THSG on platelet aggregation induced by collagen (2 μg/mL), thrombin(0.04U/mL), U46619 (3 μM) and ADP (2 μM). ATP secretion induced by collagen (2 μg/mL) was also investigated. P-selectin expression and PAC-1 binding were measured by flow cytometry. In addition, human platelet spreading on immobilized fibrinogen and immunoblotting were also tested.
Results: THSG dose-dependently inhibited platelet aggregation and ATP secretion induced by collagen. It inhibited platelet P-selectin expression and PAC-1 binding induced by thrombin(0.1U/mL). THSG also inhibited human platelet spreading on immobilized fibrinogen, a process mediated by platelet outside-in signaling. Western blot analysis showed that THSG could inhibit platelet Fc γ RIIa, Akt(Ser473)and GSK3β(Ser9) phosphorylation.
Conclusions: Our study indicates that THSG has potent anti-platelet activity to collagen induced aggregation. THSG is likely to exert protective effects in platelet-associated thromboembolic disorders by modulating human platelet.

Introduction

Platelets play important roles in hemostasis, thrombosis, wound healing, atherosclerosis, inflammation, immunity and tumor metastasis [1–3]. The main function of platelets is forming clots to stop bleeding, preventing blood loss, and maintenance of vascular integrity. This function must be under strict supervision because dysregulated thrombus formation causes ischemia. Thrombotic occlusions of coronary or cerebral arteries can lead to myocardial infarction and stroke, respectively, which are the leading causes of death worldwide [4,5]. Platelet activation and aggregation play a key role in the pathogenesis of thrombosis [6].

Currently approved anti-platelet drugs include acetylsalicylic acid, $P2Y_{12}$ antagonists, phosphodiesterase inhibitors and antagonists of the major platelet integrin αIIbβ3. However, complications are associated with the use of currently available anti-platelet drugs, and the efficacy of these drugs remains to be further improved. Thus, within the last decade or so, the investigation of novel anti-platelet targets has prospered [7,8]. Nowadays there is a special focus on natural compounds in traditional Chinese medicine.

THSG (chemical structure in Fig. 1A) is a water-soluble component of the rhizome extract from the traditional Chinese herb *Polygonum multiflorum*, which has been widely used as a tonic, lubricating intestine and anti-aging agent since ancient times. Recent studies have demonstrated that THSG possess a variety of pharmacological activities, including free radical-scavenging and anti-oxidant effects [9]. It also has been demonstrated that THSG has an anti-inflammatory effect which against experimental colitis induced by acetic acid and mitomycin C in mice [10,11]. Moreover, the polyphenolic structure of THSG is similar to that of resveratrol, which are found in red wines, has been shown to inhibit platelet activity. The low incidence of ischaemic heart disease observed in the French population is called the 'French paradox' and was partly attributed to a moderate consumption of resveratrol-containing red wine [12]. Therefore, we hypothesized THSG have the same anti-platelet effect.

Abbreviations: THSG, 2,3,5,4'-tetrahydroxystilbene-2-O-β-D glucoside; PRP, platelet rich plasma; PPP, platelet poor plasma; U46619, 9,11-Dideoxy-9a,11a-methanoepoxy prostaglandin F2a; TxA2, thromboxaneA2; ADP, adenosine diphosphate; ATP, adenosine triphosphate; PGE1, Prostaglandin E1; EDTA, ethyle nediaminetetraacetic acid; MAPK, mitogen, activated protein kinase.

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http://dx.doi.org/10.1016/j.thromres.2013.11.006

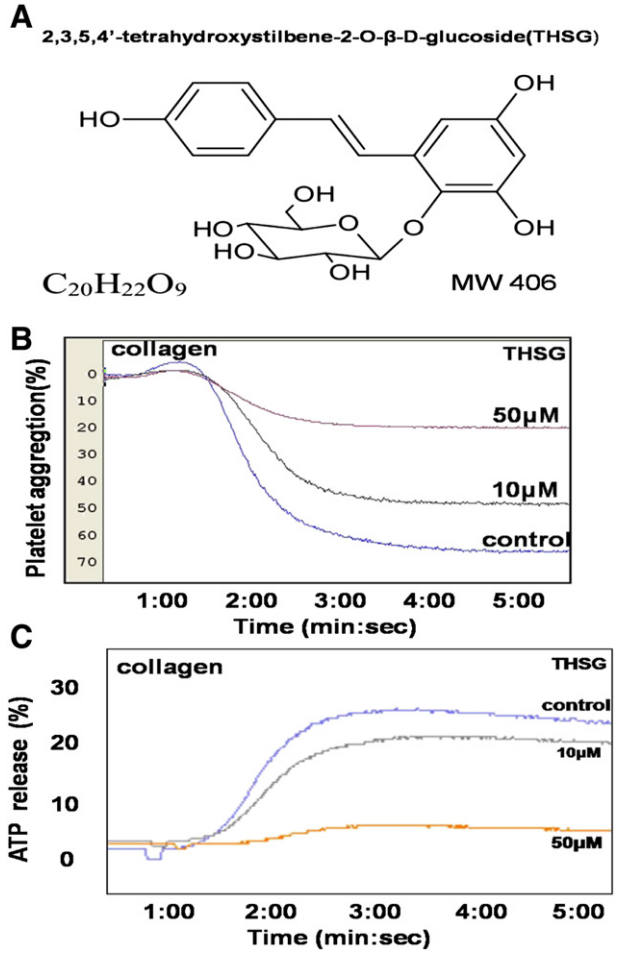

Fig. 1. (A) Chemical structure of THSG. (B and C) Inhibitory effect of THSG on agonist-induced platelet aggregation and ATP-release in human washed platelets. Washed platelets (3.0×10^8/mL) were pre-incubated with THSG(10 μM and 50 μM) or a solvent control followed by the addition of collagen (2 μg/mL) to trigger platelet aggregation(B). For ATP-release reaction experiment(C), washed platelets (3.0×10^8/mL) were pre-incubated with THSG(10 and 50 μM) or a solvent control, followed by the addition of collagen (2 μg/mL). Figures (B and C) are representative examples of at least four similar experiments.

Fc γ RIIa as the ITAM-bearing receptor mediates αIIbβ 3 outside-in integrin signaling in human platelets [13]. Akt, also known as protein kinase B, is the key downstream indicator of PI3K [14,15]. Akt phosphorylation can be used as an effector of PI3K pathway activation [16,17]. Glycogen synthase kinase 3β(GSK3β) has a potential effect in the regulation of platelet function [18]. PI3K/Akt has various downstream targets such as Bad and mTOR, but more importantly, it phosphorylates and inactivates GSK3β [19]. The activation of Akt causes the phosphorylation of GSK3 β [20].

Until recently, the anti-platelet effects of THSG and its mechanism have not been thoroughly investigated. In the present study, we examined the influence of THSG on the activities and functions of normal human platelets. We also examined the cellular signal events associated with THSG-inhibited platelet activation in vitro.

Materials and Methods

Materials

THSG (dissolved in distilled water, molecular weight 406, purity above 98%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Collagen, ATP standard and Chrono-Lume were purchased from Chrono-log Corp. (Haver-town, PA, USA). Fluorescein isothiocyanate (FITC)-conjugated antibody PAC-1 and CD62P (P-selectin) antibody were obtained from BD Biosciences (San. Jose, CA, USA). Adenosine diphosphate(ADP), prostaglandin E1(PGE1), thrombin, U46619 (thromboxane A2 analogue), and bovine serum albumin(BSA) were purchased from Sigma Chemicals (St Louis, MO, USA). The hybridoma cell line for the anti-Fc γ RIIa monoclonal antibody (mAb), IV.3, was obtained from the American Type Culture Collection (Manassas, VA). Antibodies specific for Akt were purchased from Santa Cruz Biotechnology(CA,USA); Antibodies specific for GSK3β, serine 9 -phosphorylated GSK3β, tyr416-phosphorylated Src and serine 473-phosphorylated Akt were purchased from Cell Signaling Technology(Beverly, MA, USA). The antiphosphotyrosine PY20 was purchased from Invitrogen (Carlsbad, CA).THSG was dissolved in distilled water and stored at −20 °C until use.

Preparation of Human Platelets

Healthy volunteers without history of hematological diseases such as platelet and coagulation disorder, and without taking drug that might affect platelet function during the preceding 2 weeks, were recruited for this study. Sixteen non-smoking healthy subjects (8 males and 8 females, aged 20–45 years) were recruited from the staff and students at the Tongji Medical College, Huazhong University of Science and Technology. Each volunteer first signed the inform consent, and then a 20 mL amount of blood was drawn from the volunteer. All experimental procedures were approved by the Ethics Committee for the Use of Human Subjects of Huazhong University of Science and Technology.

Whole peripheral human blood was drawn without stasis into the siliconized vacutainer tubes containing acid citrate dextrose, supplemented with 50 ng/mL prostaglandin E1 (PGE_1) and spun at 154 g for 15 minutes. Platelet-rich plasma(PRP) was collected and diluted 1:1 in Tyrode buffer(137 mM NaCl, 12 mM $NaHCO_3$, 2 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 5.5 mM glucose and 5 mM HEPES). And after the addition of 50 ng/mL PGE_1, platelets were pelleted at 750 g for 10 minutes. Platelets were washed twice in Tyrode buffer containing 50 ng/mL PGE_1 and 1 mM EDTA, pH 7.4, and finally resuspended in Tyrode buffer to a final concentration of 3.0×10^8 platelets/mL. $CaCl_2$ (1 mM) was added prior to the agonist stimulation.

Measurement of Platelet Aggregation and ATP Release Reaction

Both the platelet aggregation and release of ATP from platelets were simultaneously measured by Lumi-aggregometer Model 700 (Chrono-log, Havertown, PA, USA) [21]. Washed platelets were incubated and stabilized at 37 °C in an aggregometry sample tube with stirring at 1000 rpm for 1 minute before testing. The samples were further pretreated with THSG (10 and 50 μM) or vehicle for 5 min, and then platelet aggregation was induced by addition of collagen (2 μg/mL), thrombin (0.04 U/mL), ADP (2 μM) or U46619 (3 μM). The extent of platelet aggregation and ATP release were measured as the maximal increase of light transmission within 5 min after the addition of agonists. Platelet aggregation is expressed as percentage of light transmission (% Light Transmission) in platelet-free medium; as platelet aggregation increases, the turbidity of sample drops due to clearance of platelet in suspension, and % Light Transmission increases accordingly. The ATP released from platelets was detected by the Chrono Lume luciferase reagent according to the manufacturer's directions with gain control set at 0.05×. The final sample volume was made up to 0.25 mL for measurements.

Measurement of P-selectin Expression and PAC-1 Binding by Flow Cytometry

To study platelet expression of P-selectin (CD62P) and PAC-1 binding on activated platelets, a FACScan flow cytometer BD Biosciences (San. Jose, CA, USA) was used. Non-stirred washed platelets(3×10^8/mL)