Journal of Ethnopharmacology 192 (2016) 10–19

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Panax quinquefolium saponin combined with dual antiplatelet drugs inhibits platelet adhesion to injured HUVECs via PI3K/AKT and COX pathways

Ming-ming Wang [a], Mei Xue [a,*], Yu Miao [a], Na Kou [b], Yong-gang Xu [a], Lin Yang [a], Ying Zhang [b], Da-zhuo Shi [a,*]

[a] Center for Cardiovascular Disease, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China
[b] Beijing University of Chinese Medicine, Beijing 100029, China

ARTICLE INFO

Article history:
Received 3 December 2015
Received in revised form
2 June 2016
Accepted 7 July 2016
Available online 8 July 2016

Keywords:
Panax quinquefolium saponin
Dual antiplatelet
Endothelial cell
Platelet adhesion
Cyclooxygenase
Thrombogenesis

ABSTRACT

Ethnopharmacological relevance: Panax quinquefolium saponin (PQS) is the active component extracted from traditional Chinese medicine *Panax quinquefolius* L. and has been widely used as a supplement to dual antiplatelet drugs (DA) for treatment of coronary artery disease (CAD) for two decades; however, the efficacy of PQS combined with DA against platelet adhesion to endothelial cells (ECs), an essential step in thrombosis, remains unclear.

Aim of the study: To compare PQS combined with DA and DA alone in inhibiting platelet adhesion to injured human umbilical vein endothelial cells (HUVECs) and to explore the possible mechanisms focusing on PI3K/AKT, COX-2/6-keto-$PGF_{1\alpha}$, and COX-1/TXB_2 pathways.

Methods: HUVECs injured by oxidized low-density lipoprotein (ox-LDL) were randomly allocated into control, model, DA, PQS+DA (P+DA), LY294002 (a PI3K inhibitor)+DA (L+DA), and LY294002+PQS+DA (LP+DA) groups. HUVEC apoptosis, platelet adhesion to injured HUVECs, and platelet CD62p expression were assayed by fluorescence activated cell sorting (FACS). The concentrations of 6-keto-$PGF_{1\alpha}$ and TXB_2 in the supernatant were measured by radioimmunoassay. Protein expression of phosphorylated-PI3K, PI3K, phosphorylated-AKT, AKT, COX-1, and COX-2 in both platelets and HUVECs was evaluated by western blot.

Results: Compared to DA alone, PQS combined with DA reduced platelet adhesion to HUVECs and HUVEC apoptosis more potently, increased the concentration of supernatant 6-keto-$PGF_{1\alpha}$ and up-regulated phospho-AKT protein in HUVECs. LY294002 mitigated the effects of PQS on HUVEC apoptosis and platelet adhesion.

Conclusions: These findings show that PQS as a powerful supplement to DA, attenuated HUVEC apoptosis and improved the DA-mediated reduction of platelet adhesion to injured HUVECs and the underlying mechanisms may be associated with PI3K/AKT and COX pathways in HUVECs and platelets. PQS might provide a new complementary approach to improve the prognosis of thrombotic diseases in future.

1. Introduction

Dual antiplatelet drugs (DA), including aspirin (ASA) and clopidogrel (CLP), are the classical therapy for coronary heart disease (CHD), particularly in patients who have undergone percutaneous coronary intervention (PCI) (Hamm et al., 2011; Levine et al., 2011). Despite DA treatment, 7.4–16.5% of such patients experience thrombotic events within the first 12 months after PCI (Mehran et al., 2013; Yusuf et al., 2001). The injured endothelium triggers platelet adhesion and aggregation (Bombeli et al., 1999). Endothelial injury is considered as an initial step of thrombogenesis (Frenette et al., 1995; Nording et al., 2015). Aspirin irreversibly acetylates platelet COX-1, which induces thromboxane synthesis, to exert an anti-platelet effect (Roth et al., 1975). It also inhibits COX-2, which hinders the recovery of the injured endothelium (Guifu et al., 2003). Clopidogrel acts by blocking the platelet P2Y12 receptor specifically to irreversibly inhibit downstream PI3K/AKT signaling, which is a central pathway for healing of endothelial injury (Ahmad et al., 2006; Miyashita et al., 2003).

Most patients with coronary heart disease (CHD) can be categorized as the syndrome of Qi-Yin deficiency and blood stasis (B. Wang and Dong, 2015; D.H. Wang and Dong, 2015). *Panax*

* Corresponding authors.
E-mail addresses: meiar@126.com (M. Xue), dz_shi@163.com (D.-z. Shi).

http://dx.doi.org/10.1016/j.jep.2016.07.015
0378-8741/

quinquefolius L. (American ginseng), a perennial plant in the genus *Panax* and family *Araliaceae*, was recommended for the treatment of CHD due to its efficacies to supplement Qi and nourish Yin (Chen and Shi, 2014). *Panax quinquefolium* saponin (PQS), which is the active component extracted from the stems and leaves of *Panax quinquefolius* L., has been listed as a Chinese herbal patent drug for treatment of CHD for 20 years(Chen and Shi, 2014; Liu et al., 2014). PQS combined with DA is superior to DA alone in reducing the size of myocardial infarction in rats (D.H. Wang et al., 2015; B. Wang et al., 2015). PQS combined with routine treatment further reduces thrombotic events in CHD patients (Zhimei et al., 2010). Studies have shown that PQS alleviates endothelial injury induced by oxidized low-density lipoprotein (ox-LDL) possibly through up-regulation of PI3K/AKT signaling pathway (Lipshutz and Ghorai, 2009). However, little is known about the effect and underlying mechanism of PQS combined with DA on platelet adhesion to injured endothelium.

To mimic the pathological process and to explore the underlying mechanism of platelet adhesion to the endothelium in vitro, Bombeli established the endothelium-platelet co-culture model (Bombeli et al., 1999). In our study, we employed this model to assess the effect of DA or PQS combined with DA on platelet adhesion to endothelial cells (ECs), and to explore the underlying mechanism focusing on the PI3K/AKT and COX signaling pathways, which exist in both HUVECs and platelets.

2. Material and methods

2.1. PQS

PQS (ginsenoside Re (G-Re) $\geq$ 75%) was kindly provided by Yisheng Pharmaceutical Co, Ltd (Jilin, China; Batch No.140311), approved by the China State Food and Drug Administration (National medicine permit no. Z20030072). Our previous studies analyzed PQS with HPLC and LC-MS^n and showed that the major active compounds of PQS are G-Re, G-Rb2, G-Rd, G-Rb1, G-Rg1, G-Rb3, pseudog insenoside F11 (P-F11) (Xue et al., 2015; Yang et al., 2010).

2.2. Agents

Ox-LDL was purchased from Guangzhou Yiyuan Biotechnology Co, Ltd. (Guangzhou, China). Aspirin (ASP) and clopidogrel (CLP) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The PI3K inhibitor LY294002 was purchased from Cell Signaling Technology (Boston, MA, USA). Endothelial culture medium (ECM) was purchased from Cell Signaling Technology (Boston, MA, USA). The 6-keto-$PGF_{1\alpha}$ and TXB_2 radioimmunoassay kits were purchased from Huaying Biotechnology Institute (Beijing, China). Antibodies against CD61 and CD62p for testing platelet activation were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Antibodies against CD61 and CD144, which were used for testing platelet adhesion to HUVECs, were purchased from Life Technologies (Carlsbad, CA, USA). The PO-PRO/7-AAD apoptosis assay kit was also purchased from Life Technologies. Antibodies against p-Akt, Akt, p-PI3K, and PI3K were purchased from Cell Signaling Technology (Boston, MA, USA), and antibodies against COX-1 and COX-2 were purchased from Abcam (Cambridge, England).

2.3. Cells and groups

Human umbilical cords were obtained from healthy donors who provided informed consent (the protocol was approved by the Ethic Board of Xiyuan Hospital, China Academy of Chinese Medical Sciences). HUVECs were isolated from fresh umbilical veins using the method described by Jaffe et al. (1973) and were cultured with ECM in 60-mm culture dishes (Corning, NY, USA) at 37 °C in a humidified 5% CO_2 atmosphere. Cultured HUVECs at passage 5 were divided into 6 groups: (1) control group: cells were cultured in a 5% CO_2 incubator at 37 °C for 16 h; (2) model group: cells were exposed to ox-LDL at the concentration of 80 mg/L for 16 h; (3) DA group: cells were treated with the culture medium containing 15 μg/mL ASP and 10 μg/mL CLP for 30 min, and then exposed to 80 mg/L ox-LDL for 16 h; (4) PQS+DA (*p*-DA) group: cells were treated with the culture medium containing 160 μg/mL PQS, 15 μg/mL ASP, and 10 μg/mL CLP for 30 min, and then exposed to 80 mg/L ox-LDL for 16 h (the dose of PQS was confirmed based on prior studies (Wang et al., 2012)); (5) LY294002+DA group (L-DA): cells were incubated with LY294002 (30 μmol/L) for 1 h, and then treated with DA and ox-LDL as described for the DA group. (6) LY294002+PQS+DA (LP-DA) group: cells were incubated with LY294002 (30 μmol/L) for 1 h and then treated with PQS, DA, and ox-LDL as described for the P-DA group.

2.4. Preparation of platelets

Platelets were obtained from healthy volunteers, who provided informed consent (the protocol was approved by the Ethics Board of Xiyuan Hospital, China Academy of Chinese Medical Sciences) and had not taken any anti-platelet or anti-coagulation drugs in the month preceding the study. Blood (5 mL) was drawn from healthy volunteers using syringes containing 10% 0.11 mol/L sodium citrate and centrifuged at $150 \times g$ for 15 min to obtain platelet-rich plasma (PRP). Subsequently, the PRP was centrifuged at $1000 \times g$ for 10 min to obtain the platelet sediment. The platelets were finally resuspended in ECM to a final concentration of 1×10^8/mL.

2.5. Observation of platelet adhesion by light microscopy

After treatment of the cells with each drug and stimulation with ox-LDL, 100 μL of the platelet suspension was added to each HUVEC culture dish (platelet: HUVEC ratio, 10:1) and incubated for 5 min, followed by observation of platelet adhesion to HUVECs under a light microscope (Leica DMIRB, Wetzlar, Germany). For each sample, at least 4 randomly chosen microscopic fields were observed.

2.6. Detection of CD144/CD61-stained platelet adhesion by fluorescence activated cell sorting (FACS)

CD144 and CD61 are specific markers for HUVECs and platelets, respectively (Trappenburg et al., 2009). CD144-positive cells are HUVECs and CD61-positive cells are platelets. After incubation with platelets for 5 min, HUVECs were gently washed three times with phosphate-buffered saline (PBS) to remove unbound platelets. HUVECs together with the adherent platelets were then harvested using 0.25% trypsin and washed once with PBS. Cells were then re-suspended in PBS at 4 °C to a concentration of 1×10^6 HUVECs/mL. Antibodies against CD61 (FITC-conjugated) and CD144 (PE-conjugated) were added, referring to the manufacturer's instructions. Samples were subsequently analyzed by FACScan (NAVIOS; Beckman Coulter, Brea, CA, USA). At least 10,000 cells that stained positive for CD144 were evaluated per sample. Platelet adhesion to HUVECs was expressed as the mean fluorescence intensity (MFI) of CD61 for the entire HUVEC population (Busca et al.,2014).

2.7. Detection of PO-PRO-1/7-AAD staining by FACS to determine HUVEC apoptosis

A membrane permeability/dead cell apoptosis kit containing PO-PRO-1 and 7-AAD was used to detect HUVEC apoptosis. The