20(S)-Panaxadiol Enhances Hemostatic Effect on Activated Platelet by Affecting Calcium Signaling

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Research

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Abstract

Background: *Panax notoginseng* (Burk.) F.H. Chen has long been used to stop bleeding for hundreds of years in China. At present, only dencichine and notoginsenoside Ft1 showed the hemostatic effect. Other ingredients from *Panax notoginseng* need to be further investigated. This study evaluates the hemostatic effect of 20(*S*)-panaxadiol (PD) and reveals its mechanism.

Methods: We performed an *in vivo* study to measure PD on the hemostatic effect of mouse tail amputation and liver scratch models, and routine blood. Plasma coagulation parameters were measured using a blood analyzer. Platelet aggregation rate and adenosine triphosphate (ATP) release were analyzed by platelet aggregometer. Subsequently, degranulation marker P-selectin (CD62P), PAC-1 (activated GP IIb/IIIa receptor marker), the concentrations of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) and cyclic adenosine monophosphate (cAMP) were also assessed.

Results: PD shortened bleeding time on the mouse tail amputation and liver scratch models and mainly increased blood platelet count in the rats after subcutaneous injection of 4 h. Meanwhile, PD decreased APTT, increased FIB content, and directly induced platelet aggregation. In the absence of Ca^{2+} , PD promoted the increase of $[\text{Ca}^{2+}]_i$ and ATP, slightly increased CD62P expression and PAC-1 binding of platelets. After the addition of Ca^{2+} , PD treatment markedly promoted platelet activation by releasing ATP level, increasing CD62P expression and PAC-1 binding, and decreasing cAMP level in platelets. Besides, PD increased phosphorylation of phosphoinositide 3-kinase (PI3K), protein kinase B (PKB or Akt), and glycogen synthase kinase β (GSK3 β) in human platelets. Excitingly, PD-induced changes included platelet aggregation, a decrease of the cAMP content, and the increases of ATP, CD62P and PAC-1, which were significantly reversed by vorapaxar, which showed a similar function as thrombin.

Conclusions: PD is an essential hemostatic ingredient in *Panax notoginseng* for promoting hematopoiesis and thrombopoiesis. PD induces platelet aggregation by affecting calcium signaling and activating PI3K/Akt/GSK3 β signaling pathway, which could contribute to the new insight for the treatment of hemorrhagic disease.

Introduction

Early bleeding control is essential in metrorrhagia and metrostaxis, wound, and other bleeding disorders. Uncontrolled hemorrhage leads to anemia, even survival-threatening conditions [1, 2]. As a traditional Chinese medicine, *Panax notoginseng* (Burk.) F. H. Chen (*P. notoginseng*) is the most famous hemostatic elixir, its unique characteristics called “hemostasis without stasis” for hundreds of years in China [3]. Compendium of Materia Medica (Ben Cao Gang Mu) records that *P. notoginseng* could stop bleeding, disperse stasis blood and relieve pain, and cure all blood diseases. Yu Qiu Yao Jie records that *P. notoginseng* has the effects of heying hemostasis, dredging blood vessels, removing blood stasis, and gathering new blood. In Chinese Pharmacopeia, *P. notoginseng* possesses the function of promoting blood circulation and removing blood stasis, hemostasis, detumescence, and pain relief. It is used to treat

hemoptysis, hematemesis, epistaxis, hemaecia, metrorrhagia and metrostaxis, wound, chest and abdomen stabbing pain, swelling, and blood stasis pain from knocks and falls, and so on. At present, only dencichine [4, 5] and notoginsenoside Ft1 [6] have shown that shorten the bleeding time of mice and promote platelet aggregation, which have a significant hemostatic effect. Despite ongoing endeavors, we think still some hemostatic ingredients are undiscovered.

The characteristics of *P. notoginseng* in treating blood troubles have a bidirectional therapeutic effect both hemostatic and anti-thrombotic action [7]. At present, there are a lot of studies about ingredients of anti-thrombosis in *P. notoginseng*, for instance, protopanaxadiol-type ginsenoside Rb1 [8], Rg3 [9], Rd and Rh2 [10], which can inhibit platelets aggregation and thrombus formation. Protopanaxadiol-type ginsenosides were hydrolyzed by intestinal bacteria, acid, base, or enzymes to yield protopanaxadiol (PPD) [11, 12]. Hydrolysis can easily cause changes in C-20 hydroxyl of protopanaxadiol dehydration, cyclization, and structural transformation, and finally generate the dehydrated aglycone (panaxadiol, PD) [13, 14]. PD is considered to be a purified sapogenin of diol-type triterpenoid with a dammarane skeleton [15], and its content is 1.92 % in *P. notoginseng* [16]. In recent years, anti-cancer activities of 20(S)-PD (Fig. 1) were widely investigated [17, 18]. But, until now, few studies have shown that 20(S)-PD affects the blood system. We have found in previous study that PPD can promote the hemostatic of model bleeding rats and induce platelet aggregation by regulating calcium signaling [19]. Gao et al reported that PPD increased ADP-induced platelet aggregation [10]. Based on the characteristic of the structure with a dammarane skeleton, we speculate that 20(S)-PD plays a potential role in platelet aggregation and participates in the hemostasis process.

Hemostasis is a pivotal process that prevents blood loss after blood vessel injury. This process is tightly regulated and depends on an intricate series of events involving platelets, vascular components, and plasma coagulation factors [20]. In these processes, platelet activity is associated with the initiation of coagulation. As a consequence of vessel wall damage, subendothelial matrixes (such as collagen, von Willebrand factor (vWF), fibronectin, et al.) are exposed to the flowing blood; circulating platelets adhere to the subendothelial surfaces [21]. During this process, platelet changes its shape, releases its granule contents, and promotes the interplay between platelets and injured endothelial cells, combined with the modulation of other factors, triggers platelet aggregation and contributes to a series of events in the coagulation cascade leading to thrombin generation and fibrin clot formation that ultimately arrests bleeding [22, 23]. Moreover, extracellular Ca^{2+} entry is a crucial step in activation, shape change, and granules release of platelets [24, 25].

Thrombin formation is initiated by the exposure of tissue factors to plasma coagulation factors after disruption of the vascular endothelium [26]. Thrombin is a key enzyme in the blood coagulation cascade and a potent platelet activator. Protease-activated receptor 1 (PAR-1) and PAR4 in human were demonstrated to mediate most platelet responses to thrombin[27]. PAR1 has a higher affinity for thrombin than PAR4, and its activation results in a faster and stronger Ca^{2+} increase. PAR1 couples G12/13, Gq, and Gi/z families of heterotrimeric G proteins, thereby connecting coagulation to a host of intracellular signaling pathways [28]. Vorapaxar (SCH530348) is a small organic molecule, high affinity,

orally active, competitive PAR-1 inhibitor [29], which inhibits platelet activation. The latest studies indicate that vorapaxar reduces the risk of cardiovascular death, myocardial infarction (MI), or stroke and increases the risk of moderate or severe bleeding compared with standard of care alone in patients [30, 31].

In our studies, we detected the hemostatic effect of PD *in vitro* and *in vivo*. The results found that PD shortened the bleeding time on the mouse tail amputation and liver scratch models, influenced APTT and fibrinogen of coagulation parameters, and induced the platelet aggregation through regulating calcium signaling and PI3K/Akt/GSK3 β . PD showed an excellent hemostatic effect. Further studies showed that the hemostatic effect of PD is similar but not identical to thrombin, which is possibly weakly associated with PAR1 on platelets. After adding the vorapaxar, PD-induced activation, release, and aggregation of platelet were reversed. These findings suggest that PD shows the hemostatic effect, will benefit basic science and aid in the development of effective therapies for hematological disorders.

Materials And Methods

Materials

20(S)-panaxadiol (PD, purity $\geq 98\%$ by HPLC) was obtained from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). Hemocoagulase was obtained from Jinzhou Ahon Pharmaceutical Co, Ltd. (Liaoning, China). Prothrombin (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen (FIB) kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Luciferin/luciferase reagent and thrombin were purchased from Chrono-Log Corporation (Pennsylvania, USA). Vorapaxar, ticagrelor, and seratrodist were obtained from MedChemExpress (New Jersey, USA). Cyclic adenosine monophosphate (cAMP) ELISA kit was obtained Sino Best Biological Technology Co., Ltd. (Shanghai, China). FITC-conjugated anti-human CD62P (P-selectin) and FITC-conjugated anti-human PAC-1 were obtained BioLegend (California, USA). Fluo-3 AM Calcium indicators were purchased from Beyotime Biotechnology (Shanghai, China). Akt, p-Akt, PI3K, p-PI3K, GSK3 β , p-GSK3 β , and β -actin were purchased from Abcam (Cambridge, Britain).

Animals

Male Kunming mice (weighting 20.0 ± 2.0 g) and male Wistar rats (weighting 190.0 ± 10.0 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd (Animal license No. SCXK (Ji)-2016-0003). The animals were housed under controlled temperature ($25 \pm 1^\circ\text{C}$), relative humidity ($60 \pm 5\%$), and a 12 h light/dark cycle with ad libitum access to food and water. This experiment was approved by the Bioethics Committee of the Changchun University of Chinese Medicine and the Institutional Animal Care (Approval NO. 20190133), which was conducted based on the guideline for the use of laboratory animals.

Bleeding time measurement

The measurements of bleeding time in mouse tail amputation and liver scratch models were established, according to previous methods [32] with some modifications. Briefly, 40 male Kunming mice (20.0 ± 2.0 g) were randomly divided into 5 groups, NS group (1 % sodium carboxymethyl cellulose-normal saline), HC group (1 KU/mL hemocoagulase), and 2, 4, 8 mg/kg PD groups. After the drugs with subcutaneously injected for 4 h, mice were anesthetized with 4 % pentobarbital sodium via intraperitoneal injection (IP). In the tail amputation model, the tails of mice were transected with a sterile razor blade at the site that 10 mm apart from the tip, and then immersed in 37 °C normal saline. The bleed time was defined as the time from the start of transection to bleeding cessation. Stop time of tail bleeding over 30 s was considered as bleeding time. In the liver scratch model, the liver injury was established by scratching the left lateral lobe with a 2-mL syringe about 1 cm, to cause the liver to bleed. Then the incision was dipped with filter paper at 10 s intervals until hemostasis. All of the mice were euthanized via cervical dislocation under anesthesia at the end of each experiment.

Routine blood test

1 % CMCNa-normal saline (NS group), 1 KU/mL hemocoagulase (HC group), and 2, 4, 8 mg/kg PD were subcutaneously injected into 5 groups of Wister male rats (n=8), respectively. After 4 h treatment, the rats were anesthetized with 4% pentobarbital sodium via IP to withdrawn blood samples from the aorta abdominal, and then placed in plastic tubes with EDTA. The XT-2000i automated hematology analyzer (Sysmex Corporation, Japan) was used to detect routine blood test.

Plasma coagulation assay

Rat blood samples were withdrawn from the aorta abdominal and then placed in a 3.8 % sodium citrate vacuum tube with a blood/coagulant ratio of 9:1, and then centrifuged at 3000 rpm for 15 min to obtain plasma. Plasma mixtures with 1.45 mL plasma and 0.05 mL different concentration of PD were incubated at 37 °C for 10 min, which were used to detect PT, APTT, TT, and FIB concentration, according to the manufacturer's protocols using the H1201 automatic coagulation analyzer (Jiangsu Horner Medical Instrument Co., Ltd., China).

Platelets analysis

Human and rat washed platelets preparation

As described above, blood from healthy consented volunteers and male Wistar rats were collected into an anticoagulant tube of 3.8 % sodium citrate, respectively. Platelet-rich plasma (PRP) was isolated as the supernatant from centrifugation at 800 rpm for 5 min. Human/rat washed platelets were prepared as before [33]. PRP was centrifuged at 3,000 rpm for 5 min and washed twice with Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO_3 , 5 mM HEPES, 0.35 % BSA, PH 7.4) to obtain human/rat washed platelets.

Platelet aggregation assay and ATP release assay

Human/rat washed platelets were adjusted to 3×10^8 /mL with Tyrode's buffer including 1mM CaCl_2 . After the incubation at 37 °C for 5 min, the platelets were stimulated by various concentrations of PD or thrombin, respectively. Platelet aggregation was performed by using a platelet aggregometer (Chrono-Log 700, Chrono-Log Co., USA) by measuring the changes in light transmission. ATP release was measured using luciferin/luciferase reagent (Chrono-lume). Additionally, vorapaxar (VP, a PAR-1 antagonist of thrombin, 10 μM), ticagrelor (TG, a P_2Y_{12} receptor antagonist of ADP, 10 μM) and seratrodoast (ST, a potent and selective thromboxane A2 receptor antagonist, 10 μM) were used to further analyze the possible mechanism of PD on platelet activity by platelet aggregation assay.

P-selectin secretion and glycoprotein (GP) IIb/IIIa activation on the surface of platelets by flow cytometric analysis

Human washed platelets were incubated with different concentrations of PD at 37 °C for 5 min, and then incubated with FITC-conjugated CD62P (P-selectin marker) or FITC-conjugated PAC-1 (activated GP IIb/IIIa receptor marker) antibodies in the dark for 20 min. After stopping by adding 200 μL of phosphate-buffered saline (PBS), the samples were immediately analyzed with a BD FACS Aria II flow cytometer (BD Biosciences, USA). A total of 10,000 events in triplicated from different groups were analyzed the platelet P-selectin secretion and glycoprotein IIb/IIIa activation, which was repeated at least three times to ensure reliability.

Determination of the intracellular calcium concentration $[\text{Ca}^{2+}]_i$

As previously reported [34], human washed platelets were incubated with Fluo-3 AM (5 μM) at 37 °C for 60 min in the dark condition, and washed two times and suspended in Tyrode's buffer. Platelets at the final concentration of approximately 3×10^8 /mL were added to the 96-well microplates (Nunc F96, ThermoFisher Scientific, Waltham, USA) and incubated with PD. After adding PD, Fluo-3 fluorescence was determined at 17 seconds intervals for 20 min on Cell Imaging Multi-Mode Reader (Cytation 5, BioTek, Vermont, USA) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm to draw calcium kinetic curve. The $[\text{Ca}^{2+}]_i$ is calculated by the previous method [35] as follows: $[\text{Ca}^{2+}]_i$ in cytosol = $525 \text{ nM} \times (F - F_{\min}) / (F_{\max} - F)$, where 525 nM is the dissociation constant of the Fluo-3, F represents the fluorescence value of the sample. $F_{\min}$ and $F_{\max}$ are minimum and maximum fluorescence values, are measured after the treatment with 10 mM EGTA and 0.1 % Triton X-100, respectively.

Measurement of cAMP

Human washed platelets were incubated with methanol or PD at 37 °C for 10 min, and then added the 10 mM EDTA to terminate the reaction. After freezing at -80°C and thawing at 37 °C for 5 times, the solution was centrifuged at 3,000 rpm for 10 min at 4 °C, and the supernatant for detecting the concentrations of cAMP using the ELISA kit according to the manufacturer's protocol. To evaluate whether the cAMP production was involved in VP inhibited the platelet aggregation. Human washed platelets were pretreated with VP (10 μM) for 5 min, then treated with PD to detect the cAMP concentration.

Western blot

To assess the effect of PD on the downstream signaling pathway of PAR1, western blot experiments were performed. The washed human platelets were pretreated with methanol or PD at 37 °C for 15 min, lysis buffer (PRO-PREP; iNtRON Biotechnology, Seoul, Korea) with 100 µL RIPA buffer and protease/phosphatase inhibitor cocktail (Beyotime Biotechnology, Shanghai, China) was added to the mixture. Thirty micrograms of the cellular proteins were resolved by electrophoresis in 10 % SDS-polyacrylamide gel, and subsequently transferred to polyvinylidene difluoride (PVDF) membrane. Following 1 h incubation in a fresh TBS buffer containing 0.1 % Tween-20 and 5 % BSA, the blots were probed with specific antibodies including p-Akt, Akt, p-PI3K, PI3K, p-GSK3β, GSK3β and β-actin overnight at 4°C. After incubation with appropriate secondary antibodies for 1 h at room temperature, protein bands were visualized and analyzed using a chemiluminescent imaging system (FluorChem, ProteinSimple, San Jose, CA, United States)

Statistical analysis

Data from all experiments are presented as the mean ± standard deviations (SD). Ordinary one-way ANOVA of variance was used to analyze the differences among the groups by GraphPad Prism 8.0 software. $p < 0.05$ was considered as statistical significance.

Results

Effect of PD on bleeding time

To detect whether PD had the hemostatic effect, tail amputation and liver scratch models in mice were performed [32, 36]. The results showed that hemocoagulase (HC) and PD treatment significantly decreased the tail bleeding time (Fig. 1B) and liver bleeding time of mice (Fig. 1C). In the mouse tail amputation, the tail bleeding time of mice in 4 and 8 mg/kg of PD groups was remarkably reduced compared with the NS group ($p < 0.05$ or $p < 0.001$, Fig. 1B). In the liver scratch model, the liver bleeding time of mice treated with PD was significantly lower than that of the NS group ($p < 0.05$ or $p < 0.001$, Fig. 1C). These results showed that PD had a good hemostatic effect on mice after subcutaneous injection 4 h, which was similar to hemocoagulase.

Effect of PD on routine blood test in rat

PD absorbed into the blood after rat subcutaneous injection of 4 h; parameters of routine blood can directly reflect the effect of PD on blood. Fig. 2 showed that the parameters of red blood cell (RBC) and platelet (PLT) had significant changes in the rat treated with PD for 4 h; and PD had no effect on the other parameters of routine blood test, such as white blood cell counts, hemoglobin, neutrophils, lymphocyte (Data are not shown). For the RBC parameters, compared with the NS group, red cell distribution width-standard deviation (RDW-SD, Fig. 2B) and red cell distribution width-coefficient of variation (RDW-CV, Fig. 2C) were increased by PD at 2 and 4 mg/kg groups ($p < 0.05$ or $p < 0.01$).

Importantly, PD remarkably increased rat PLT counts (Fig. 2D), plateletcrit (PCT, Fig. 2E), and platelet larger cell ratio (P-LCR, Fig. 2F) compared with the NS group ($p<0.05$ or $p<0.01$). These results showed that PD mainly affected the parameters of RBC and PLT in the rats after subcutaneous injection of 4 h.

Effect of PD on coagulation parameters in rat

To further determine the hemostatic effect and the mechanism of PD, rat plasma was used for detecting coagulation parameters such as APTT, PT, TT, and FIB concentration as important indicators. Compared with the vehicle group, the clotting time was significantly shortened by PD (70 and 140 μM) in the APTT assay (Fig. 3A, $p<0.05$); However, PT and TT had no difference between vehicle group and PD treatment groups (Fig. 3B and 3C, $p>0.05$). FIB concentration markedly increased in three PD-dose groups (Fig. 3D, $p<0.05$ or $p<0.01$). APTT reflects the endogenous coagulation system [37], and FIB is an acute-phase protein in the last step of hemostasis [38]. So above results suggested that PD showed hemostatic effects by activating endogenous coagulation system and accelerating FIB transformed into fibrin.

PD promoted human/rat washed platelet aggregation

Platelets play a crucial role in primary hemostasis and wound healing [25]; PD had demonstrated hemostatic effects by *in vivo* experiment study. Therefore we examined the effect of PD on platelet aggregation and its mechanism. As shown in Fig. 4A and 4B, with the increase of PD concentration, the human platelet aggregation rate increased dramatically; the platelet aggregation rate was 36.00% at 280 μM . Fig. 4C and 4D showed the same effect that PD induced washed rat platelet aggregation. With the increase of PD concentration, the rat platelet aggregation rate remarkably increased; the max rat platelet aggregation rate was about 51.40 % at 280 μM (Fig. 4D). Obviously, the aggregation effect of PD on human/rat platelets was very weaker than that of thrombin on human/rat platelets. These results indicated that PD could directly induce platelet aggregation of human and rat; the aggregation effect of PD on human platelets was weaker than that on rat platelets.

PD activated platelets by promoting calcium influx, releasing granule and increasing the GP IIb/IIIa expression

Calcium signaling is the common platelet activation signaling pathway [39]. The elevation of intracellular Ca^{2+} contributes to several events of platelet activation, such as shape change, granule release, and GP IIb/IIIa activation [40]. PD on the human platelets calcium kinetic curve was detected by applying Cell Imaging Multi-Mode Reader. Fig. 5A showed that PD could only moderately increase the concentration of Ca^{2+} in platelets along with the time; however, thrombin markedly instantaneously increased the Ca^{2+} influx into platelets. As shown in Fig. 5B, 140, 70, and 35 μM PD could significantly increase the concentration of Ca^{2+} after 5 min, 10 min, and 15min, respectively ($p<0.05$). Platelets are stimulated by agonists to release granules including α -granules and dense granules that further activate platelets. ATP (dense granule) release and P-selection (CD62P, α -granule) expression are commonly used as a marker to quantify the level of platelet activation [41]. Fig. 5C showed that PD increased the ATP concentration, but had no significance compared with the vehicle group ($p>0.05$). Fig. 5D showed that PD significantly

increased the CD62P expression rate compared with the vehicle group, which showed a dose-dependent effect.

Platelet aggregation is the result of fibrinogen binding to its platelet receptor, GP IIb/IIIa. PAC-1 is a monoclonal antibody that is used to measure the expression of GP IIb/IIIa complex [42]. PAC-1 binding was studied to explore platelet aggregation further. As Fig. 5E shown, PAC-1 binding rate of washed human platelets treated with PD significantly increased compared with the vehicle group ($p<0.001$), the max binding rate was 43.85 % at 140 μM .

Importantly, platelet activation stimulated by various agonists is strongly dependent on the increased Ca^{2+} concentration in the cytoplasm [39]. Furthermore, we observed the effect of PD on platelet activation in the Ca^{2+} -dependence. As shown in Fig. 6A, after adding 1 mM Ca^{2+} , PD dose-dependently increased ATP release of human washed platelets compared with the vehicle group ($p<0.01$ or $p<0.001$); however, ATP release of platelet without 1mM Ca^{2+} was weaker than that of platelet with 1mM Ca^{2+} (Fig. 5C). Fig. 5D showed that PD promoted the CD62P expression rate of human washed platelets when the concentration of PD was 140 μM , CD62P expression rate was 35.80 % ($p<0.001$); after adding 1 mM Ca^{2+} , CD62P expression rate of PD group remarkably increased compared with the vehicle group that reached 84.32 % at 140 μM PD (Fig. 6B). Similarly, PD significantly increased the PAC-1 binding rate of human washed platelets with 1 mM Ca^{2+} and arrived at 140 μM , which was dose-dependent and similar to that of thrombin (Fig. 6C). The above results showed that alone PD could weaker activation of platelets, which did not directly induce platelet aggregation (Data are not shown); adding large amounts of Ca^{2+} enhanced the activation of PD on platelets, and accelerated the platelets aggregation (Fig. 4).

VP inhibited PD-induced human platelet activation, release and aggregation

To further identify the molecular mechanism of PD on platelet activation, we use inhibitors (such as VP, TG, and ST) screening of potential sites of action, Fig. 7 showed PD-induced human platelet aggregation was markedly inhibited by VP ($p<0.001$), but TG and ST did not affect PD-induced platelet aggregation (Fig. 7A and 7B). VP is a PAR-1 antagonist of thrombin [30]. Our results showed that PD (140 μM)-induced human platelet aggregation might be mediated by regulating PAR-1 [43]. Therefore, we further detect the effect of different concentrations of PD combined with VP on platelet aggregation. As can be seen from Fig. 7C, with the increase of PD concentration, the human platelet aggregation rate increased; after adding the VP, the platelet aggregation rates notably reduced. VP did not wholly inhibit PD-induced human platelet aggregation. When the concentration of PD was 140 μM , the maximum inhibition rate of VP was 49.28% (Fig. 7C) on human platelet aggregation.

Further development, we detected ATP release, CD62P expression rate, and PAC-1 binding rate of PD combined VP on platelets. Fig. 7 showed that PD-induced increases of ATP, CD62P, and PAC-1 were markedly inhibited by VP (Fig. 8A, $p<0.001$, Fig. 8B, $p<0.001$ and Fig. 8C, $p<0.001$). The above results showed that PD induced platelet activation, release and aggregation partly through regulating PAR-1 pathway.

Effect of PD on downstream signaling pathways of PAR1

PAR1 is coupled to G_i proteins that lead to a reduction in cAMP [44], and $G\beta\gamma$ -mediated activation of PI3K that plays an essential role in the platelet activation [45, 46]. To assess the effect of PD on the signaling pathway of PAR1, we examined cAMP level, and phosphorylation of downstream intracellular signaling molecules including PI3K, the serine/threonine kinase Akt and GSK3 β in platelets. As shown in Fig. 9, compared with the vehicle group, PD could markedly inhibit cAMP production of human platelets, which showed a dose-dependent manner (Fig. 9A, $p < 0.05$, $p < 0.01$ or $p < 0.001$). The combination of PD with VP reversed PD-induced the reduction of cAMP concentration in human platelets (Fig. 9B, $p < 0.05$). Furthermore, Fig. 10 showed that PD markedly increased phosphorylation of PI3K, Akt and GSK3 β molecules in a dose-dependent manner. These results indicated that PD might activate downstream proteins of PAR1, such as cAMP, PI3K, Akt and GSK3 β , to promote the platelets aggregation; and VP inhibited the progress of platelet aggregation.

Discussion

Many factors participate in the hemostasis process after vessel wall damage, such as the capillary function, tissue shrinkage ability, count and function of cell factor and platelets, function of the fibrinolytic system of human or mice [20]. In this study, all of the results affirmed the hemostatic effect of PD of *P. notoginseng* by activating the endogenous coagulation system and inducing platelet aggregation.

First, we detected that PD stopped bleeding time at mouse tail amputation and liver scratch models *in vivo*. The results showed that PD had a good hemostatic effect on bleeding models of the mouse (Fig. 1). Second, the effect of PD on rat routine blood test was evaluated. The results showed that PD increased remarkably RBC and PLT parameters (Fig. 2). Increases of RBC-SD and RBC-CV reflect the changes in the hetero normocytic population [47], which often appear in cardiovascular disease [48]. Platelets play an important role in the course of hemostasis and thrombosis under physiological or pathological conditions [23]. PCT provides more comprehensive data about total platelet mass [49]; high HCT value associated with coronary artery disease [50]. P-LCR is the measure of larger platelets; some reports been shown that large platelets are biologically more active and their prothrombotic properties are more powerful [51, 52]. Increases of platelet counts, PCT and P-LCR indicate platelet reactivity increased that have a prothrombotic tendency [51, 53]. RBC-SD, RBC-CV, PLT counts, HCT, and P-LCR of serum in rats were increased by PD, which indicated that PD could promote hematopoiesis and thrombopoiesis [54]. RBC and PLT participated in the hemostatic process to accelerate thrombosis at the site of bleeding with the PD treatment group after rat subcutaneous injection 4 h. Activated platelets secrete an abundance of granules to maintain and amplify the initial response of the platelets and stimulate more circulating platelets that are recruited to aggregates at the injured vessel wall [41]. And RBC is compressed to close-packed polyhedral structures with platelets and fibrin on the surface in contracted clots and thrombi [55]. The variation of the indexes as mentioned above indicated that PD could accelerate the hemostasis on blood vessel or other tissue wounds *in vivo*.

To further confirm the coagulation pathways of PD, coagulation parameters were carried out *in vitro*. APTT and PT are sensitive and commonly used screening test for intrinsic and extrinsic coagulation systems, respectively [37]. APTT reflects the level of coagulation factor VIII, IX, X, XI, and XII in plasma; PT reflects the overall activity of coagulation factor III, VII, V, and X in plasma [37, 56]; TT primarily reflects whether there is an abnormal level of fibrinogen, anticoagulant and fibrinolytic substance in the common pathway of coagulation process that fibrinogen converted to fibrin [57]. At the end of these processes, fibrinogen is transformed into fibrin, which transformed the blood from the collosol state to the gel state [38]. In this study, the decrease of APTT and the increase of FIB content suggest that PD had good hemostatic effects by activating the endogenous coagulation system to accelerate the formation of FIB.

In vivo experiment studies demonstrated that PD had hemostatic effects; we further examined the effect of PD on platelet aggregation and explored its mechanism. Activated platelets release dense granules (such as 5-HT, ADP, ATP, histamine, CD63, etc.) and α -granules (P-selectin, PF4, vWF, and thrombospondin-1, etc.) [41], which modulate the function of interacting platelets and blood vascular cells. Some granules such as ATP, ADP, 5-HT, vWF stimulate additional circulating platelets that are recruited to form the aggregates. Our results had shown that PD activated the platelet, and increased ATP content and CD62P expression of human platelets to induce platelet aggregation (Fig. 4 and 5). Additionally, GP IIb/IIIa is exposed on platelets surface to enable the binding of soluble ligands and activation-dependent changes in the conformation of GP IIb/IIIa can be detected by specific antibody (PAC-1) [58]. PD increased the PAC-1 binding rate of activated platelets, which promoted fibrinogen into insoluble fibrin and participated in the hemostatic process (Fig. 5).

Human platelets maintain a low resting $[Ca^{2+}]_i$ estimated to be around 50~100 nM [59]. An increase in platelets $[Ca^{2+}]_i$ is a pivotal signaling event during platelet activation. PD markedly increased $[Ca^{2+}]_i$ of human platelets after 5 min of incubation, which was slower and more extended time than thrombin (Fig. 5). Ca^{2+} signaling participated in the regulation of platelet activation, shape change, granule release, thrombus formation, and GP IIb/IIIa activation [40, 60]. In the absence of Ca^{2+} , PD promoted the GP IIb/IIIa activation (PAC-1 binding rate) and CD62P expression, which was very weaker than that of thrombin (Fig. 5). When 1 mM Ca^{2+} was added to platelets, PAC-1 binding rate, CD62P expression and ATP release of platelets were noticeable rises treated with PD (Fig. 6). These results showed that the high concentration of Ca^{2+} enhanced the activation of PD on platelets and collectively accelerated platelet aggregation (Fig. 4). In other words, PD induced the platelet aggregation that might be dependent on the high concentration of Ca^{2+} .

The platelet membrane has many receptors, such as GPIb/V/IX, GPVI, $\alpha_2\beta_1$, PARs, $P2Y_1$, $P2Y_{12}$, thromboxane A2 receptor (TP), and integrins [58]. VP, TG, and ST are anti-platelet agents through antagonism of PAR-1, $P2Y_{12}$, and TP, respectively [61-63], which were used to screen the potential sites of PD on platelets. The results revealed that only VP could inhibit PD-induced platelet aggregation (Fig. 7). PAR1 and PAR4 of human platelet were demonstrated to mediate most platelet responses to thrombin [27]. PAR1 can activate heterotrimeric G proteins of the G12/13, Gq, and Gi/z families [64, 65] to impact a

coagulation-related network of signaling pathways [28]. The α -subunits of G13 bind rho guanine nucleotide exchange factors (RhoGEFs) to be involved in shape changes in platelets [66, 67]. $G\alpha_q$ involves stimulation of PLC β that results in increased intracellular Ca^{2+} and activation of protein kinase C [68, 69]. These provide a pathway to calcium-dependent kinases and phosphatases, RhoGEFs, mitogen-activated protein kinases and other proteins that mediate cellular responses ranging from granule secretion, integrin activation, and aggregation in platelets [28, 70]. Stimulation of receptors coupled to G proteins containing a $G\alpha_i$ subunit result in the $G\beta\gamma$ -mediated activation of PI3K [46]. Both PLC β and PI3K mediate secretion, calcium response, and aggregation in platelets, which play vital roles in platelet cytoskeletal dynamics [71]. And $G\alpha_i$ -mediated inhibition of adenylylate cyclase reduces the level of cAMP in platelets [44]. In this study, PD had a similar interaction site of platelets as thrombin and might weaker bind to PAR1 in platelets to slowly promote platelet aggregation, which was inhibited by VP (Fig. 7). Meanwhile, VP reversed the results that PD stimulated platelets to release dense granules (P-selection) and increase PAC-1 binding rate (Fig. 8), and the reduced level of cAMP in platelets induced by PD was returned to normal level after adding VP (Fig. 8C). The PI3K/Akt/GSK3 pathway has emerged as a major signaling axis regulating platelet responses [72]; PKC and Akt modulate platelet function by phosphorylating and inhibiting GSK3 α/β on thrombin-mediated platelet activation [73]. Our results had shown that PD increased the phosphorylation level of PI3K, Akt, and GSK3 β (Fig. 10). All of the above results showed that PD induced platelet aggregation that may mainly be involved in PAR1 pathway.

In conclusion, PD, an aglycone of protopanaxadiol-type ginsenosides, directly induced platelet aggregation and promoted blood hemostasis. The hemostatic effect of PD was dependent on calcium signaling and PI3K/Akt/GSK3 β signaling pathway. PD is a critical ingredient of *P. notoginseng* for hemostatic effect, which might act as hemostatic medicine for clinical therapy of hemorrhage.

Abbreviations

PD: 20(*S*)-panaxadiol; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; PT: Prothrombin; APTT: activated partial thromboplastin time; TT: thrombin time; FIB: fibrinogen; HC: hemocoagulase; PRP: platelet-rich plasma; VP: vorapaxar; TG: ticagrelor; ST: seratrodist; GP: glycoprotein; PAR-1: Protease-activated receptor 1; RBC: red blood cell; PLT: platelet; RDW-SD: red cell distribution width-standard deviation; RDW-CV: red cell distribution width-coefficient of variation; PCT: plateletcrit; P-LCR, platelet larger cell ratio.

Declarations

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No applicable.

Author's contributions

HZ[#] and WS[#] contributed equally to this work. DZ conceived and designed the study. HZ and BQ wrote the main manuscript text. WJ, XT, DP and YY performed animal experiments. CY analyzed and collected data. XL revised the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This experiment was approved by the Bioethics Committee of the Changchun University of Chinese Medicine and the Institutional Animal Care (Approval NO. 20190133), which was conducted based on the guideline for the use of laboratory animals.

Consent for publication

We declare that the Publisher has the Author's permission to publish the relevant contribution.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The effect of PD on the mouse bleeding time. (A) The chemical structure of 20(S)-panaxadiol (PD); (B) Tail bleeding time was measured in tail amputation model of mice treated with normal saline (NS), hemocagulase (HC) or PD (2, 4 and 8 mg/kg) for 4 h; (C) Liver bleeding time was measured in liver scratch model of mice treated with NS, HC or PD (2, 4 and 8 mg/kg) for 4 h. The data are expressed as mean $\pm$ SD (n=8). * $p < 0.05$ and *** $p < 0.001$ compared to the NS group.

Figure 2

The effect of PD on rat blood routine. A-C, The effect of PD on red blood cell parameters, including red blood cell (RBC) counts (A), red cell distribution width-standard deviation (RDW-SD, B), and red cell distribution width-coefficient of variation (RDW-CV, C). D-F, PD on platelet-related parameters, platelet (PLT) counts (D), plateletcrit (PCT, E), and platelet larger cell ratio (P-LCR, F) were measured after subcutaneously injected 4 h in rats of NS, HC and PD (2, 4 and 8 mg/kg) groups. The data are expressed as mean $\pm$ SD (n=8). *p<0.05 and **p<0.01 compared to the NS group.

Figure 3

The effect of PD on coagulation parameters of rat plasma in vitro. (A-C) After incubation for 10 min at 37 °C, 200 μL of the rat plasma mixture with PD was blended with APTT (200 μL), PT (150 μL), or TT (150 μL) assay reagents to detect APTT, PT or TT, respectively. The clotting times (s) was recorded immediately and monitored using an automatic coagulation analyzer. (D) FIB. After incubation for 10 min at 37 °C, 200 μL of rat plasma mixture with PD was blended with 100 μL thrombin assay buffer to record clotting time (s) immediately. The standard curve was drawn based on the concentration of fibrinogen (x, g/L) and clotting time (y, s) ($y = -0.1505x + 57.363$) for determining the content of FIB. The data are expressed as mean $\pm$ SD (n=3). *p<0.05 and **p<0.01 compared to the vehicle group.

Figure 4

PD induced human/rat washed platelets aggregation. (A, C) Human or rat platelets aggregation was observed, after the stimulation with methanol or PD (35, 70 or 140 μ M); (B, D) (C, F) Methanol, thrombin (0.5 U/mL) or different concentrations of PD (17.5, 35, 70, 140 or 280 μ M) induced the increase of human or rat washed platelet aggregation rate. After the incubation of human washed platelets (290 μ L) with 1 mM Ca^{2+} for 5 min at 37 $^{\circ}\text{C}$, different concentrations of PD (10 μ L) were added and shaken at 1,200 rpm/min to detect the platelets aggregation. The data are expressed as mean $\pm$ SD (n=3). **p<0.01 and ***p<0.001 compared to the vehicle group.

Figure 5

PD activated platelets without external Ca²⁺. (A) The calcium kinetic curves of the human platelets were stimulated by PD. Human washed platelets were incubated with Fluo-3 AM (5 μ M) at 37 °C for 60 min in the dark conditions, platelets loaded with Fluo-3 was washed two times and suspended in Tyrode's buffer to a final concentration of approximately 3 $\times$ 10⁸ platelets/mL, 140 μ L Fluo-3-loaded platelets were added to the 96-well microplates. After adding 10 μ L menthol, thrombin (0.5 U/mL) and PD (35, 70 and 140 μ M), Fluo-3 fluorescence was recorded at 18 seconds intervals for 20 min using Cell Imaging Multi-Mode Reader with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. (B) The effect of menthol, PD and thrombin on Ca²⁺ concentration ([Ca²⁺]_i) of human platelets at 5 min, 10 min, 15 min and 20 min, respectively. (C) The effect of PD on ATP release of platelets. 290 μ L human washed platelets were incubated at 37°C for 5min, and then added the 10 μ L menthol, thrombin or different concentration of PD and 30 μ L LUME Reagent (Chrono-lume) to detect the ATP release using a platelet aggregometer. (D) The effect of PD on P-selectin (CD62P) of human washed platelets. (E) The effect of

PD on PAC-1 of human washed platelets. (D, E) 145 μ L human washed platelets were incubated with 5 μ L menthol, thrombin (0.5 U/mL) or PD (35, 70, 140 μ M) at 37 $^{\circ}$ C for 5 min, and then added into anti-CD62P FITC or anti-PAC-1 FITC in the dark for 20min. After stopping by adding 200 μ L of PBS, the samples were immediately analyzed with a flow cytometer. The data are expressed as mean $\pm$ SD (n=3). *p<0.05, **p<0.01 and ***p<0.001 compared to the vehicle group.

Figure 6

PD activated platelets with 1 mM Ca²⁺. (A) The effect of PD on ATP release of human washed platelets with 1mM Ca²⁺. 290 μ L human washed platelets were incubated at 37 $^{\circ}$ C for 5 min, and then added 10 μ L menthol, thrombin (0.5 U/mL) or PD (35, 70, 140 μ M) and 30 μ L LUME Reagent (Chrono-lume) to detect the ATP release using a platelet aggregometer. (B) The effect of PD on the CD62P expression of human washed platelets with 1 mM Ca²⁺. (C) The effect of PD on the PAC-1 binding rate of human washed platelets with 1 mM Ca²⁺. (B, C) 145 μ L human washed platelets were incubated with 5 μ L menthol, thrombin (0.5 U/mL) or PD (35, 70, 140 μ M) at 37 $^{\circ}$ C for 5 min, and then added into anti-PAC-1 FITC or anti-CD62P FITC in the dark for 20 min. After stopping by adding 200 μ L of PBS, the samples were immediately analyzed with a flow cytometer. The data are expressed as mean $\pm$ SD (n=3). **p<0.01 and ***p<0.001 compared to the vehicle group.

Figure 7

The effect of three inhibitors on PD induced the platelets aggregation. (A) Light transmission graphs of PD combined with VP, TG and ST on human washed platelet. (B) The aggregation rate in human platelets of PD alone or combined with VP, TG, or ST. (C) Light transmission graphs of PD with VP, TG and ST on rat washed platelet. (D) The aggregation rate in human platelets of PD alone or combined with VP, TG, or ST. (E) VP inhibited different concentrations of PD induced human washed platelet aggregation. (F) VP inhibited different concentrations of PD induced rat washed platelet aggregation. (A, B, C and D) 290 μ L human/rat washed platelet with VP (10 μ M), TG (10 μ M) or ST (10 μ M) were stimulated by 10 μ L PD (140 μ M) at 37 $^{\circ}$ C with shaking at 1200 rpm/min, using a platelet aggregometer recorded the platelet aggregation. (E and F) 290 μ L human/rat washed platelet with or without VP (10 μ M) were stimulated by 10 μ L different concentrations of PD (0, 17.5, 35, 70, 140, 280 μ M); platelet aggregation was measured by using a platelet aggregometer at 37 $^{\circ}$ C with shaking at 1200 rpm/min. The data are expressed as mean $\pm$ SD (n=3). ns was no significant between the two groups. ###p<0.001 compared to the thrombin group, ***p<0.001 compared to the PD group.

Figure 8

VP inhibited ATP release, CD62P expression rate and PAC-1 binding rate of PD induced human washed platelets. (A) VP inhibited ATP release rate of PD stimulated human platelets with 1 mM Ca²⁺. 290 μ L human washed platelets were incubated at 37°C for 5min, and then added the 10 μ L menthol, thrombin (0.5 U/mL) or PD (140 μ M) and 30 μ L LUME Reagent (Chrono-lume) to detect the ATP release using a platelet aggregometer. (B) VP inhibited the CD62P expression rate of PD stimulated human platelets with 1 mM Ca²⁺. (C) VP inhibited the PAC-1 binding rate of PD induced human washed platelets with 1 Mm Ca²⁺. (B and C) 145 μ L human washed platelets with 10 μ L VP (10 μ M) were incubated with 5 μ L thrombin (0.5 U/mL) or PD (140 μ M) at 37 °C for 5 min, and then added into anti-CD62P FITC or anti-PAC-1 FITC in the dark for 20min. After stopping by adding 200 μ L of PBS, the samples were immediately analyzed with a flow cytometer. The data are expressed as mean $\pm$ SD (n=3). ***p<0.001 compared to the two groups without and with VP.

Figure 9

The effects of PD on cAMP of human and rat platelets. (A) The effect of PD on the cAMP concentration of human washed platelets. (B) The effect of PD on the cAMP concentration of rat washed platelets. (C) VP inhibited the decrease of the cAMP content of PD caused human platelets. (A-B) Human/rat washed

platelets were incubated with menthol, thrombin (0.5 U/mL), PD (35, 70, 140 μ M) at 37 $^{\circ}$ C for 10 min, and then added the 10 mM EDTA to terminate the reaction. After freezing at -80 $^{\circ}$ C and thawing at 37 $^{\circ}$ C for 5 times, the solution was centrifuged at 3,000 rpm for 10 min at 4 $^{\circ}$ C, and the supernatant for detecting the concentrations of cAMP using the ELISA kits. (C) Human washed platelets were pretreated with VP (10 μ M) for 5 min, then treated with PD (140 μ M) following the same procedure described above. The data are expressed as mean $\pm$ SD (n=3). ns was no significant between the two groups. *p<0.05, **p<0.01 and ***p<0.001 compared to the vehicle group, #p<0.05 and ##p<0.01 compared to the two groups without and with VP.

Figure 10

PD increased phosphorylation of PI3K, Akt and GSK3 β in human platelets. The data are expressed as mean $\pm$ SD for 3 determinations. *p<0.05, **p<0.01 and ***p<0.001 compared to vehicle group.

Supplementary Files

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