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INVESTIGATION AND CHARACTERIZATION OF THE ANTIPLATELET ACTIVITIES OF PHYSALIN B

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Abstract*- *Physalin B is a steroidal triterpenoid whose effects on platelet functions are not clearly understood. This paper presents novel in vitro and ex vivo studies on the effects of physalin B on platelet activation and thrombosis formation. Peripheral blood mononuclear cells were cultured to obtain two types of endothelial progenitor cells from the same donors. MTT and cell adhesion assays were performed to evaluate cell viability and the adhesion of THP-1 cells to human umbilical vein endothelial cells (HUVECs), respectively. Biomarkers included prothrombin time (PT) and activated partial thromboplastin time (APTT). The experimental results indicated that physalin B can inhibit the activation of human platelets through an ex vivo interfering arachidonic acid pathway and can block the activation of P2Y12 receptors. Moreover, the antiplatelet function of physalin B does not affect plasma coagulation because both PT and APTT remain unchanged after treatment. Furthermore, physalin B at 80 μM can reduce the TNF-α-induced adhesion of monocytic leukocyte THP-1 cells to HUVECs in a cell adhesion assay. These results suggest that the antithrombotic activity of physalin B is attributed to its antiplatelet activation and antiinflammatory effects. To the best of our knowledge, this study is the first to report on the antiplatelet effect of secosteroidal physalin-type compounds.

Keywords - physalin B; platelet activation; anticoagulation

I. INTRODUCTION

Activated platelets stimulate thrombus formation in response to atherosclerotic plaque rupture or endothelial erosion, thereby promoting atherothrombotic events. Activated platelets also interact with endothelial cells and leukocytes to promote inflammation, which contributes to atherosclerosis. Therefore, antiplatelet drugs are important in cardiovascular disease therapy. Clopidogrel, a thienopyridine, combined with aspirin is the current "gold standard" for reducing cardiovascular events in patients with acute coronary syndrome (ACS). However, not all patients optimally respond to this standard therapy. When used either alone or in combination, resistance to the antiplatelet activity of both drugs occurs, possibly leading to treatment failure including additional atherothrombotic events [1]. Furthermore, the risk of bleeding is always a major clinical concern when these antiplatelet therapies are applied. Thus, development of a more effective and reliable new drug for antiplatelet aggregation is necessary.

Platelets are involved in many physiological and pathological processes, such as atherosclerosis, stem cell trafficking, tumor metastasis, and arthritis. Platelet activation at sites of an intact inflamed endothelium contributes to vascular inflammation and vascular wall remodeling. Platelets interact with the vascular endothelium and link the processes of inflammation, thrombosis, and atherogenesis, which is mediated through the interactions between platelets and endothelial cells/leukocytes [2–4]. Platelets can induce a variety of inflammatory responses in monocytes, neutrophils, endothelial cells, or endothelial progenitor cells (EPCs), resulting in key inflammatory processes, such as adhesion, chemotaxis, migration, thrombosis, or even monocytic cell differentiation to macrophages or foam cells [4]. EPCs are pluripotent cells that can differentiate into mature endothelial cells. Previous studies have demonstrated that healthy persons have a small number of circulating EPCs in the peripheral blood [5,6]. In recent years, investigations have focused on elucidating the cellular mechanisms of EPCs on vasculogenesis to find new methods to alleviate certain cardiovascular disease conditions [5,7,8]. The level of circulating EPCs was considered as a marker to predict the prognosis of acute coronary events [9], and served as a biological index for vascular function and cardiovascular risk.

Physalin B is a natural secosteroidal triterpenoid from the *Physalis angulata* plant, a member of the Solanaceae family. It has been demonstrated to possess antiinflammatory activities in macrophages through a marked inhibition of NO

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production [10]. Recently, physalin B has been shown to inhibit hemocytic activity by depressing the insect platelet-activating factor analog (iPAF) levels. In our previous studies, we reported that physalin B significantly suppressed the survival of melanoma A375 and A2058 cells, but was less cytotoxic for skin fibroblast CCD-966SK, T/G HA-VSMC, and H9c2 cells [11]. In the present study, we investigated the effects of physalin B on platelet function using the PFA-100 and *VerifyNow* systems. Our study demonstrated that physalin B inhibited the aspirin reaction units (ARU) and P2Y12 reaction units (PRU) in a dose-dependent manner. Physalin B does not interfere with the coagulation pathways because prothrombin time (PT) and activated partial thromboplastin time (APTT) are essentially the same in platelet-poor plasma treated with physalin B. Meanwhile, using the MTT assay, we found no adverse effects for physalin B on the growth of human umbilical vein endothelial cells (HUVECs) or late EPCs. Our results suggest that physalin B can be used as a potent antithrombotic agent, and that the antithrombotic activity may be because of its antiplatelet activity.

II. RESULTS AND DISCUSSION

2.1. Physalin B displayed no obvious cytotoxicity on platelets

Before performing a series of experiments on antiplatelet aggregation assays, we tested the cytotoxic effect of physalin B on platelets via the lactate dehydrogenase (LDH) release assay. The amount of LDH released from platelets after treatment with various concentrations of physalin B for 30 min at 37°C were measured. No obvious LDH release can be detected with the use of up to 160 μM physalin B (Fig. 1). These results clearly indicate that physalin B (up to 160 μM) will not induce cytotoxic effects against platelets.

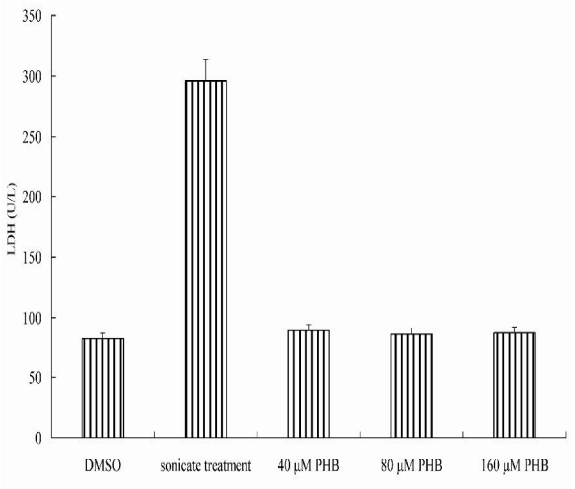

Figure 1. Physalin B showed no cytotoxicity to platelets. The cytotoxic effect of physalin B (PHB) on platelets was tested via the lactate dehydrogenase (LDH) release assay. The platelets were exposed to various concentrations of physalin B, as labeled on the x-axis, or DMSO as the vehicle. The final concentration of DMSO in the test medium was <0.1%. Sonication physically ruptured the platelets and served as a positive control. Tests for each concentration of physalin B were performed 3 times.

2.2. Physalin B displayed no obvious cytotoxic activity on HUVECs and EPCs

To evaluate the cytotoxicity of physalin B against other cell types under nearly physiological conditions, we set up an EPC primary culture and HUVEC culture and examined the effect of physalin B. EPCs not only resemble *in vivo* vascular endothelial cells, their vasculogenesis potentials also urge us to investigate if physalin B will have any inhibitory effects on them. We cultured the cells from human peripheral blood to get late EPCs, as described in detail in experimental section .Two different types of EPCs from a source of adult peripheral blood were obtained and labeled early EPCs and late EPCs, according to their time-dependent appearance. Briefly, mononuclear cells (MNCs) were first isolated from peripheral blood and subsequently plated on 6-well tissue culture plates precoated with human fibronectin. Small colonies appeared after 1-2 weeks in a culture of MNCs. The initially seeded cells were round in shape (Fig. 2A). After 5 to 10 days, attached cells appeared in clusters (Fig. 2B and 2C). In 2 to 4 weeks after plating (Fig. 2D), these cells had a more smooth cytoplasmic outline, were firmly attached to the plate (Fig. 2E), and had a cobblestone appearance similar to HUVECs when they divided. These cells rapidly replicated from several cells to form a colony and became late EPCs, which were a monolayer with almost full confluence (Fig. 2F).

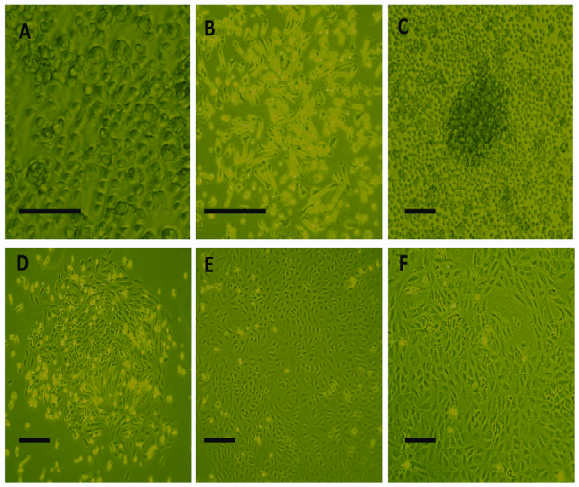

Figure 2. Morphology and characterization of late EPCs. The EPC primary culture was established from mononuclear cells (MNCs) isolated from human peripheral blood. Cells were plated on 6-well tissue culture plates precoated with human fibronectin. Scale bar is 100 um. (A) The initially seeded cells. (B) 5, (C) 10, (D) 20, and (E) 30 days after plating. The cells become more attached to the plate and clustered over time. (F) Confluent monolayer of late EPCs, 4 weeks after initial seeding, subcultured 3 times.

Furthermore, we examined the *in vitro* cytotoxic activity of physalin B at different concentrations (0, 20, 40, 80, and 160 μM) against HUVECs and EPCs. A standard MTT assay was used for this purpose. The time-dependent and dose-dependent growth inhibition of physalin B in the above cell lines are shown in Figure 3. Although HUVECs and late EPCs were both susceptible to physalin B at relatively high concentration (40 μM) and long incubation time (48 h), late

EPCs were much less vulnerable to the cytotoxic effect of physalin B. Physalin B showed a much lower toxicity against EPCs than HUVECs after 48 h of treatment, with IC_{50} values of 76 and 30 μM, respectively (Fig. 3A and B). These results clearly indicate that the cytotoxicity of physalin B is mild for HUVECs and EPCs, revealing no apparent adverse effects on their functions.

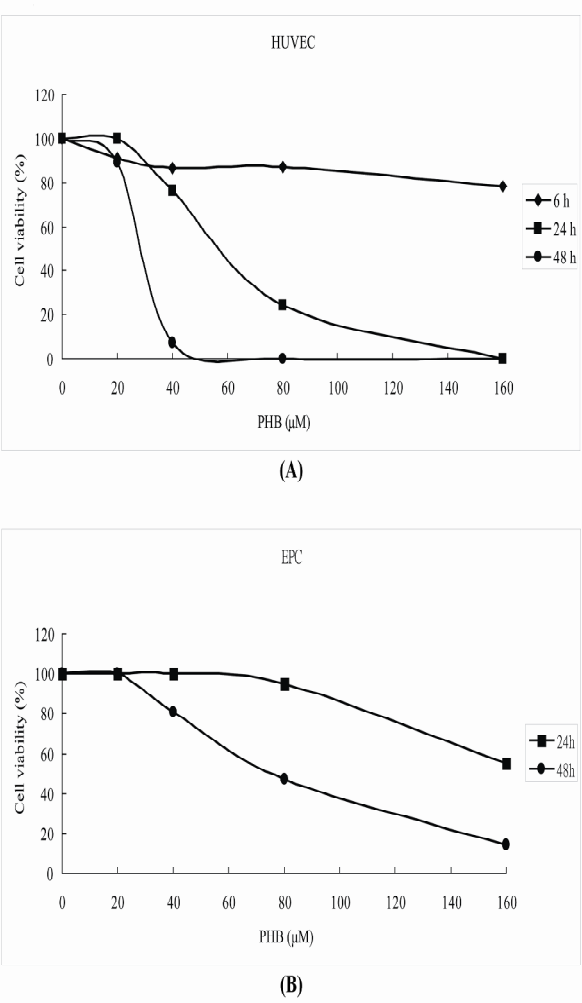

Figure 3. Effect of physalin B on HUVEC and EPC viability. Cell viability was measured using the MTT assay. After the MTT assay, the cell viability was normalized to the control MTT reduction rate, which was set as 100% viable. (A) Viability of HUVECs after treatment with indicated physalin B concentration for 6 h, 24 h, and 48 h. (B) Viability of EPCs after treatment with indicated physalin B concentration for 24 h and 48 h. The final concentration of DMSO in the test medium was <0.1%. Each point was performed in triplicate.

2.3. Physalin B inhibits platelet aggregation without affecting plasma coagulation activity

We then examined the effect of physalin B on platelet functions, using three assays. First, we applied the PFA-100 system to measure platelet aggregation, which monitors platelet plug formation through closure of a simulated aperture.

Furthermore, we used the *VerifyNow* system to differentiate the effects of physalin B on established platelet activation pathways. Finally, we engaged Sysmex CA-1500 to assess the influence of physalin B on coagulation factors. Whole blood samples of all volunteers had normal values, and the vehicle alone (0.5% DMSO) had no effect on the PFA-100 closure time. In both collagen-epinephrine and collagen-ADP-triggered plug formation conditions, pretreating samples with 10 μM physalin B exhibited statistically significant prolonged closure time. The inhibitory effect was more profound in the case of collagen-epinephrine as the stimulus (Fig. 4), with a closure time twice as long as the control conditions (125 s for the control and 260 s for physalin B treatment). These results demonstrate that physalin B can affect platelet aggregation, and thus prevent plug formation.

We then attempted to determine the pathways that are affected by physalin B upon platelet activation. The *VerifyNow* system can distinguish the activation of platelets through arachidonic acid, P2Y12, or glycoprotein (GP) IIb/IIIa pathways. In contrast to the negative controls, the aspirin reaction unit (ARU) values obtained from blood samples treated with either 20 or 50 μM physalin B were both below the 550 criteria (ARU = 469 and 366, respectively), and were similar to that of therapeutic effective aspirin treatment (ARU = 404, Table 1). For inhibition of the P2Y12 receptor activation pathway, physalin B demonstrated mild effects at 20 μM, but significantly inhibited this pathway at 50 μM, as the P2Y12 reaction unit (PRU) fell far below the normal range (PRU = 65, Table 1). Ticagrelor and clopidogrel were used as positive controls for this study. Ticagrelor reversibly inhibits the platelet P2Y12 receptors, which results in rapid inhibition of platelet activation and aggregation.

Clopidogrel also acts on this receptor; however, because it is a prodrug, the inefficiency in transformation to the active metabolite in whole blood tends to result in slower and less consistent inhibition of platelets. The value of physalin B at 20 μM (PRU = 226) was similar to clopidogrel treatment (PRU = 263); however, we do not currently know if any form of physalin B metabolites can have a stronger inhibitory effect on this pathway. Furthermore, because P2Y12 is the receptor for the ADP activator, the inhibitory effect of physalin B on the P2Y12 pathway is consistent with its interference on collagen-ADP induced platelet aggregation (Fig. 4). Although profound effects on arachidonic acid and P2Y12 pathways were detected, physalin B apparently did not interfere with GPIIb/IIIa receptor activity.

Physalin B treated samples yielded similar platelet aggregation unit (PAU) values as the negative control (PAU = 144 and 161, respectively). This is in contrast to the potent, synthetic, nonpeptidic GPIIb/IIIa receptor antagonist aggrastat (PAU = 1, Table 1). From this, we concluded that physalin B can modulate the two major upstream platelet activation pathways triggered by arachidonic acid and ADP, but has little or no effect on the later platelet aggregation event; namely, binding of platelets to fibrinogen through GPIIb/IIIa receptors.

TABLE 1. TEST OF ANTIPLATELET FUNCTION USING THE VERIFYNOW SYSTEM. THE DEGREE OF AGGREGATION FOR THE ARACHIDONIC ACID PATHWAY WAS REPORTED IN ASPIRIN REACTION UNITS (ARU). P2Y12 RECEPTOR ACTIVATION WAS REPORTED IN P2Y12 REACTION UNITS (PRU). THE PLATELET GLYCOPROTEIN (GP) IIB/IIIA RECEPTOR ACTIVITY WAS REPORTED IN PLATELET AGGREGATION UNITS (PAU)

Samples	Aspirin (ARU)	P2Y12 (PRU)	GPIIb/IIIa (PAU)
No treatment	660	314	161
Aspirin	404	-	-
Ticagrelor	-	1	-
Clopidogrel	-	263	-
Aggrastat	-	-	1
20 μM Physalin B	469	226	144
50 μM Physalin B	366	65	129

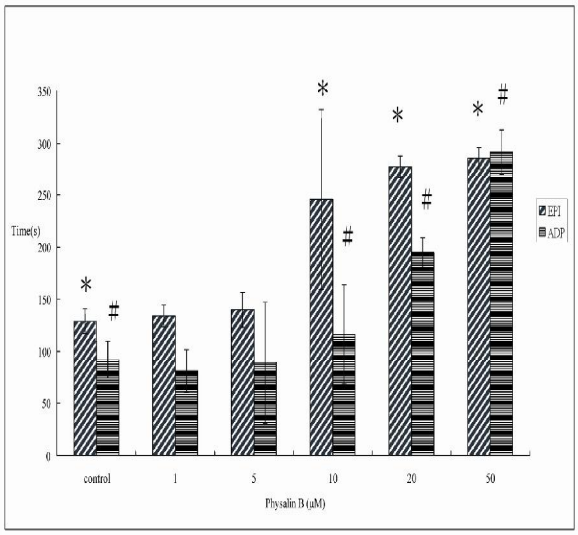

Figure 4. Effect of physalin B on platelet aggregation. In this experiment, the closure time required for platelets to plug an aperture after platelet activation was measured using PFA-100, as described in the experimental section. EPI: activated by collagen and epinephrine; ADP: activated by collagen and ADP. Data were obtained from 10 healthy blood samples, and * and # indicated $P < 0.05$ by t-test compared with the control values.

To investigate the interactions between physalin B and coagulation factors, we evaluated the effects of physalin B on coagulation time by PT, APTT, and a fibrinogen assay using platelet-poor plasma. All of the coagulation parameters tested were not affected by physalin B treatment at concentrations of up to 50 μM (Table 2). For the fibrinolytic activity assay, a normal fibrinogen level was maintained with physalin B treatment, indicating that physalin B will not deplete effective fibrinogen for blood coagulation. These results suggest that physalin B likely has no undesirable effects on plasma coagulation.

TABLE 2. EFFECTS OF PHYSALIN B ON HUMAN PLASMA COAGULATION TIMES. THE RESULTS ARE EXPRESSED AS THE MEAN + SD (N = 6). PT: PROTHROMBIN TIME, APTT: ACTIVATED PARTIAL THROMBLASTIN TIME, N.C.: NO COAGULATION. TIME IS EXPRESSED IN SECONDS

Samples	Concentration	PT (s)	APTT (s)	Fibrinogen (mg/dL)
Saline	0	10.6 ± 0.1	30.2 ± 0.9	231.1± 17.5
PHB	20 μM	10.5 ± 0.5	30.6 ± 2.7	234.7 ± 40.5
PHB	50 μM	10.5 ± 0.4	30.4 ± 2.5	233.6 ± 39.5
Heparin	120 U	99.7 ± 2.3	N.C.	163.2 ± 20.5

2.4. Physalin B reduces adhesion of THP-1 cells to HUVEC monolayers

Furthermore, we attempted to investigate the effect of physalin B on monocytic leukocyte and endothelial cell interactions. Current studies suggest that interactions between monocytes, platelets, and vascular endothelial cells possibly initiate inflammation events, and are a key step in atherogenesis. Therefore, we evaluated the influence of physalin B on the adhesion of monocyte cell line THP-1 to HUVECs under inflammatory conditions by a cell adhesion assay. We used a vital fluorescence calcein-AM staining technique to monitor the *in vitro* adhesion of THP-1 cells to HUVECs upon addition of TNF-α to stimulate the inflammatory process. Calcein-AM is nonfluorescent and permeable to cell membranes, but only viable cells can cleave its acetoxymethyl (AM) ester group, resulting in strong fluorescence emission at 525 nm. THP-1 cells were labeled with calcein-AM first, then cocultured with TNF-α and physalin B pretreated HUVECs. After a wash procedure, the fluorescence intensity remaining in the culture was proportional to the number of THP-1 cells attached to HUVECs. Here we only incubated HUVECs with physalin B for 6 h to avoid the cytotoxicity of physalin B during prolonged incubation; however, this is still sufficient to start the cell adhesion event. The fluorescent cells can be either visualized under a fluorescent microscope or quantified using a fluorescence reader. Treatment of TNF-α exposed cells with 80 μM physalin B inhibited THP-1 cell adhesion to HUVECs by approximately 35%, which is statistically significantly lower than TNF-α treatment alone ($P < 0.05$) (Fig. 5). This revealed that physalin B also possesses antiinflammatory effects through the inhibition of monocytic leukocyte adhesion to epithelial cells.

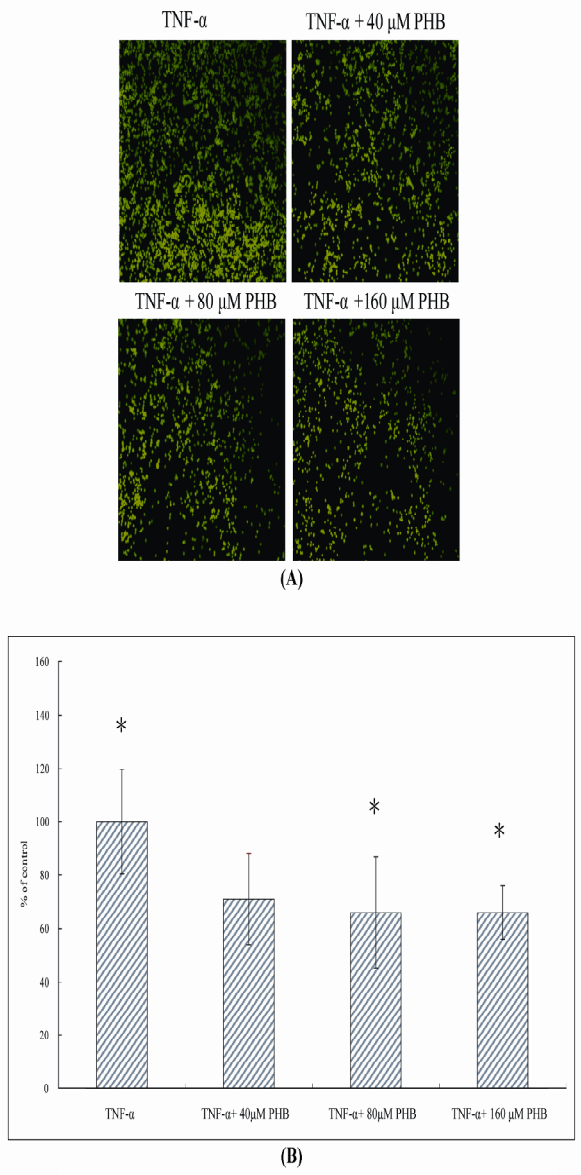

Figure 5. Adhesion of calcein-labeled THP-1 monocytes to TNF-α-activated HUVECs was inhibited by physalin B. HUVECs activated by 10 ng/mL TNF-α were treated with the indicated concentration of physalin B and then cocultured with calcein-labeled THP-1 monocytes, as described in the experimental section. (A) Represented microphotographs were obtained using a fluorescence microscope. (B) Fluorescence emission measured from THP-1 cells attached to HUVECs. Data are from experiments performed in triplicate and expressed as a percentage of adhesion to TNF-α-induced HUVECs. Values are expressed as the mean± SD.* $P < 0.05$ compared with TNF-α alone.

2.5. Discussion

Physalin B is a strong inhibitor of the ubiquitin-proteasome pathway, and this property is responsible for its inhibition of the NF-kB cascade [12]. NF-kB is the main transcriptional activator for a number of endothelial cell adhesion molecules responsible for inflammation-induced leukocyte adhesion [13]. In contrast, the platelet-activating factor (PAF) is an important activator of the NF-kB pathway [14], and a previous study reported that physalin B had no significant effect on phospholipase A2 (PLA2) activities; however, a significant enhancement of PAF-acetyl hydrolase (PAF-AH) in *R. prolixus* was observed [15]. One study demonstrated that physalins may also have antiinflammatory activities in macrophages, as assessed by a marked inhibitory action on NO production [10]. Physalin B also possesses immunomodulatory activities, as demonstrated by its ability to inhibit allogeneic transplant rejection in mice [16]. In the present study, we not only demonstrated that physalin B can inhibit adhesion of monocytic leukocytes to HUVECs, an initial step in inflammation, we also, for the first time, discovered that physalin B can inhibit platelet activation and aggregation. These two activities make physalin B a potential lead drug for atherosclerosis prevention and cardiovascular therapy.

Platelet activation plays an important role in the process of inflammation and the initiation of atherosclerosis. Many cardiovascular diseases (CVDs), including the initiation of atherosclerosis, are linked to the abnormal and excessive activation of platelets, or platelet hyperactivity, which is considered an independent risk factor for CVDs [17]. Acetylsalicylic acid (aspirin) was the first antiplatelet agent identified, which irreversibly inhibits the cyclooxygenase 1 (COX1) enzyme in the arachidonic acid pathway through acetylation of the COX1 active site. Long-term aspirin therapy reduces the risk of subsequent myocardial infarctions, strokes, or vascular death among intermediate to high-risk patients with atherothrombotic disease by about 20-25% [18]. However, the risk of bleeding is a substantial limitation of antiplatelet therapy. In contrast, thienopyridines (ticlopidine and clopidogrel) target platelet activation pathways critical for both protective hemostasis and pathologic thrombosis, which can be detected clinically as a prolonged bleeding time [19]. Though recent novel antiplatelet agents, including clopidogrel and ticagrelor, provide potent antiplatelet effects on CVD therapy, bleeding remains an important clinical issue. The balance between bleeding and efficacy for a safe antiplatelet agent are still being investigated. In the present study, we discovered that physalin B could inhibit platelet aggregation in human blood stimulated by either collagen–epinephrine or collagen-ADP, and did not show any fibrinolytic activity or alter the coagulation parameters. These properties make physalin B a potential alternative drug for CVD therapy. However, whether *in vivo* administration of physalin B causes prolonged bleeding time requires further studies since aspirin, ticagrelor, and aggrastat also did not interfere with the function of coagulation factors . What will be the potential target for physalin B in platelet activation pathways? Since physalin B effectively inhibited the arachidonic pathway, cyclooxygenase is a likely target for physalin B. However, the pathways activated by ADP, epinephrine, and collagen converge at G proteins, which in turn activate phospholipase C (PLC). PLC generates IP_3, which further opens the calcium channel. Whether physalin B could affect PLC or the calcium channel is currently unknown, and physalin B acting alone or in combination is worth further study.

Adult bone marrow (BM) is a rich reservoir of tissue-specific stem cells and progenitor cells. Among these, a scarce population of cells described as EPCs can be mobilized into circulation by various stimuli to contribute to

neoangiogenic processes or repair the damaged endothelial cell layer. Emerging evidence suggests that EPCs play a crucial role in neovascularization of ischemic tissue and contribute to reendothelialization of injured blood vessels [20,21]. Circulating EPCs are either early EPCs or late EPCs. The early EPCs have low proliferative capacity and fail to form vessels. In contrast, late EPCs have high proliferative potential and play a crucial role in neoangiogenesis [22,23]. In our previous study, physalin B induced cell death in melanoma cancer cell lines but not in nontumor cells [11]. In the present study, it was also demonstrated that physalin B was only mildly cytotoxic to HUVECs and late EPCs.

An early step in atherosclerosis is the adhesion of monocytes to the arterial wall, followed by infiltration and differentiation into leukocytes. The leukocyte adhesion includes rolling, adhesion, firm adhesion, and finally transmigration through endothelial cell junctions, which prompt the progression of atherosclerosis [24]. This key step is mediated by the interaction of leukocytes with adhesion molecules expressed by endothelial cells. In the current study, we tested the hypothesis that physalin B could modulate leukocyte adhesion to HUVECs. Our results demonstrated that physalin B reduced leukocyte adhesion to endothelial cells. This finding is consistent with those of the previous study on the antiinflammatory effect of physalin B, and inhibited the reperfusion-induced increase in vascular permeability, recruitment of leukocytes in the intestine and lung, and intestinal hemorrhaging [25]. In addition, this could also be beneficial to the prevention of atherosclerosis.

The leaf of the *Physalis angulata* plant exhibits a very bitter taste and is used for liver protection in Taiwanese traditional medicine. Physalins, particularly the major product physalin B, are regarded as the active compounds in this plant. Our study is the first to show the antiplatelet effect of physalin B. We reported that physalin B significantly inhibits human platelet activation without attenuating coagulation factors. From the *ex vivo* study on antiplatelet function, we further demonstrated that the major effects were derived from inhibition of the arachidonic acid pathway and P2Y12 receptor activation. The current study suggests that physalin B may play a vital role in protecting against CVDs, including decreasing platelet-mediated inflammation and atherosclerosis. The mechanisms of these inhibitory effects, particularly the direct effect of physalin B on platelet signaling pathways, merit further study.

III.EXPERIMENTAL SECTION

Lactate dehydrogenase assays

Cytotoxicity of physalin B on platelets was evaluated by measuring the release of lactate dehydrogenase (LDH). Platelets were suspended in Tyrode's solution at a concentration of 3×10^8 platelets/mL. For the positive control, platelets were disrupted by sonication at 4°C for 30 s at 40 kHz with a Biosonic Sonicator. The supernatant was used for LDH determinations. LDH activity was measured as an increase in the absorbance of NADH at 340 nm using lactate as the substrate. The tests were conducted with a Toshiba Medical automatic chemical analyzer, TBA-200FR (Toshiba Medical products). The platelets were pretreated with various concentrations of physalin B for 30 min at 37°C, and the supernatant was used to measure the LDH activity.

Cell culture of endothelial progenitor cells

Two different types of EPCs were cultured from adult peripheral blood and defined as early EPCs and late EPCs, according to their time-dependent appearance. Late EPCs showed a long lifespan and rapidly proliferated and are considered mature endothelial cells. Peripheral blood (20 mL) was obtained from donors with informed consent. The mononuclear cells were fractionated from other components of peripheral blood by centrifugation (Histopaque 1077, Sigma-Aldrich, St. Louis, MO, USA), with gradients according to manufacturer instructions. The isolated mononuclear cells were resuspended with an EGM-2 BulletKit system (catalog number CC-3162; Clonetics) consisting of an endothelial basal medium, 5% fetal bovine serum, hEGF, VEGF, hFGF-B, IGF-1, ascorbic acid, and heparin; 1×10^7 mononuclear cells per well were seeded on 2% gelatin-coated 6-well plates (Sigma-Aldrich, St. Louis, MO, USA) and incubated in a 5% CO_2 incubator at 37°C. Under daily observation, the first media change was performed approximately 5 days after plating. Thereafter, media were changed every 3 days. Each cluster or colony was checked every day. For all assays, late EPCs were used at passages 3-5.

MTT assay for cell viability

Cell viability was measured with blue formazan that was metabolized from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Amresco USA) in mitochondria, which is active only in live cells. HUVECs and late EPCs were seeded in a 96-well plate at a density of 1×10^5 cells per well, cultured overnight, and pretreated with various concentrations of physalin B. After incubation for 6 h, 24 h, and 48 h, the MTT (5 mg/mL) colorimetric viability test was used to determine the viability of the cells. The absorbance of each well was measured at 540 nm with an ELISA reader, and the percentage viability was calculated.

Determination of platelet function and anticoagulation assay

Ten healthy, nonsmoking volunteers (six women, four men), aged 23-39 years, participated in this study. The citrated whole blood samples were incubated at room temperature for 60 min with either physalin B (dissolved in 0.5% DMSO) or the vehicle alone. Platelet function measurements were performed with a PFA-100 analyzer system (Dade-Behring, Marburg, Germany). The PFA-100TM device measures the closure time (CT) required for platelets to plug an aperture, simulating an injured vessel after platelet activation by relevant stimuli; namely, collagen-epinephrine (EPI) or collagen-ADP (ADP). The maximum value for closure time is 300 s, and values >300 s are reported as nonclosure. The closure time was determined with duplicate samples of 800 µL using cartridges containing collagen-epinephrine or collagen-ADP membranes.

To further identify the platelet surface receptors that are involved in the antiplatelet effect, the *VerifyNow* system (Accumetrics, San Diego, CA, USA) was applied. It is a whole-blood assay based on light transmission measurements. The assay is a turbidimetric-based optical detection system, which like optical aggregometry, depends on the ability of activated platelets to bind fibrinogen. When platelets are activated, they form aggregates with fibrinogen-coated beads,

and the light transmission through the samples is increased. Arachidonic acid, ADP, and thrombin receptor activating peptide are the corresponding agonists used in the *VerifyNow* system to specifically differentiate the platelet activation pathways. In the aspirin-specific assay, the degree of aggregation attenuated by aspirin, which blocks the arachidonic acid pathway, is quantified according to a corresponding decrease in light transmission, and is reported as aspirin reaction units (ARU). An ARU value <550 indicates adequate platelet inhibition by aspirin treatment. The residual activity of platelets after inhibition on the P2Y12 receptor is represented as P2Y12 reaction units (PRU). The normal PRU distribution range without antiplatelet therapy is 194-418. Usually, we use a P2Y12 antagonist, such as clopidogrel or ticagrelor, as the positive control. The residual platelet activity under the treatment of antagonist on glycoprotein (GP) IIb/IIIa receptors was reported as platelet aggregation units (PAU). The reference range was 125-330. Aggrastat is often used as the positive control medication for PAU value.

We performed activated partial thromboplastin time (APTT), prothrombin time (PT), and fibrinogen with 3.2% sodium citrate plasma on a fully automated coagulation analyzer (Sysmex CA-1500, Sysmex, Japan) after physalin B or heparin treatment. Platelet-poor plasma was first incubated with physalin B or heparin at 37°C for 7 min. Extrinsic factor activity (II, V, VII, X), intrinsic factor activity (VIII, IX, XI, XII), and fibrinogen concentration were measured with a one-stage prothrombin time-based assay (PT, Siemens, PT Innovin), one-stage activated partial thromboplastin-based assay (APTT, Siemens, Actin FSL), and Clauss Method (Siemens, Dade thrombin reagent), respectively.

Assay for THP-1 cell adhesion to HUVECs

HUVECs were starved in serum-free medium for 1 h before treatment. For adhesion assays, HUVEC monolayers in 96-well plates were treated for 2 h with physalin B and/or TNF-α for 4 h. After treatment, 1×10^6 THP-1 cells labeled with 5 μM calcein-AM were seeded onto confluent HUVECs and cocultured for 30 min at 37°C in a 5% CO_2 incubator. Nonadherent THP-1 cells were removed by washing with phosphate buffered saline (1 × PBS) twice. Cell images were collected using a fluorescence microscope (Zeiss) and quantified using a fluorescence microplate reader at an excitation wavelength of 490 nm and an emission wavelength of 525 nm (Bio-Tek Synergy HT).

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