

Original Article

Genipin inhibits endothelial exocytosis *via* nitric oxide in cultured human umbilical vein endothelial cells

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Aim: Exocytosis of endothelial Weibel-Palade bodies, which contain von Willebrand factor (VWF), P-selectin and other modulators, plays an important role in both inflammation and thrombosis. The present study investigates whether genipin, an aglycon of geniposide, inhibits endothelial exocytosis.

Methods: Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured. The concentration of VWF in cell supernatants was measured using an ELISA Kit. P-selectin translocation on the cell surface was analyzed by cell surface ELISA. Cell viability was measured using a Cell Counting Kit-8. Mouse bleeding times were measured by amputating the tail tip. Western blot analysis was used to determine the amount of endothelial nitric oxide synthase (eNOS) and phospho-eNOS present. Nitric oxide (NO) was measured in the cell supernatants as nitrite using an NO Colorimetric Assay.

Results: Genipin inhibited thrombin-induced VWF release and P-selectin translocation in HUVECs in a dose- and time-dependent manner. The drug had no cytotoxic effect on the cells at the same doses that were able to inhibit exocytosis. The functional study that demonstrated that genipin inhibited exocytosis *in vivo* also showed that genipin prolonged the mouse bleeding time. Furthermore, genipin activated eNOS phosphorylation, promoted enzyme activation and increased NO production. L-NAME, an inhibitor of NOS, reversed the inhibitory effects of genipin on endothelial exocytosis.

Conclusion: Genipin inhibits endothelial exocytosis in HUVECs. The mechanism by which this compound inhibits exocytosis may be related to its ability to stimulate eNOS activation and NO production. Our findings suggest a novel anti-inflammatory mechanism for genipin. This compound may represent a new treatment for inflammation and/or thrombosis in which excess endothelial exocytosis plays a pathophysiological role.

Keywords: exocytosis; genipin; human umbilical vein endothelial cells; P-selectin; von Willebrand factor
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Introduction

Exocytosis of endothelial cell granules is one of the earliest responses to injury or stimulation of vascular issue and plays an important role in both inflammation and thrombosis^[1,2]. The granules in endothelial cells are termed Weibel-Palade bodies (WPB) and contain pro-inflammatory and pro-thrombotic proteins such as the von Willebrand factor (VWF), P-selectin, and other vascular modulators^[3–5]. Endothelial cells secrete WPBs in response to a number of inflammatory secretagogues^[1,3,4]. When endothelial cells

are stimulated, the membranes of WPBs rapidly fuse with the endothelial plasma membrane, releasing the WBP contents into the space outside of the endothelial cells^[3,4]. The proteins released by WPB exocytosis promote neutrophil and platelet adhesion to vessel walls and cause vascular inflammation^[3,5–7]. Therefore, inhibition of endothelial cell exocytosis can decrease inflammation and vascular thrombosis.

Genipin is a metabolite of geniposide, the major active ingredient of *Gardenia jasminoides* Ellis fruit, which has long been used in traditional Chinese medicine^[8–10]. Genipin has anti-inflammatory^[9,11], anti-diabetic^[12], anti-thrombotic^[13], anti-oxidative^[9,14], anti-angiogenic^[9] and neurotrophic activities^[15]. In a variety of animal models, genipin has anti-inflammatory activities. It significantly inhibited

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acute inflammation in carrageenan-induced rat paw edema, carrageenan-induced rat air pouch edema and croton oil-induced mouse ear edema models^[9,11]. Genipin also inhibited the changes in mouse vascular permeability induced by acetic acid^[11]. Genipin may inhibit inflammation, in part by inhibiting the expression of inducible nitric oxide synthase (iNOS), by inhibiting the production of nitric oxide (NO) upon stimulation by lipopolysaccharide or interferon in a murine macrophage cell line, as well as by inhibition of nuclear factor κ B (NF- κ B) activation^[9]. However, the molecular mechanisms of the anti-inflammatory action of genipin are still not fully understood. Because endothelial exocytosis plays an important role in vascular inflammation, we hypothesized that genipin would exert its anti-inflammatory effect by inhibiting WPB exocytosis from endothelial cells.

In this study, we investigated the effects of genipin on VWF exocytosis and P-selectin translocation in primary cultures of human umbilical vein endothelial cells (HUVECs). We also performed experiments to further examine the mechanisms responsible for the inhibitory effect of genipin on endothelial exocytosis.

Materials and methods

Reagents Genipin (98%, Figure 1), thrombin, collagenase, Endothelial Cell Growth Supplement (ECGS) and 3,3',5,5',-tetramethylbenzidine substrate solution (TMB) were purchased from Sigma-Aldrich (St Louis, MO, USA). Endothelial cell basal medium (EBM) and fetal calf serum (FCS) were from Gibco (Grand Island, NY, USA). Penicillin was from Invitrogen (San Diego, CA, USA). Goat polyclonal antibodies to P-selectin and to phospho-eNOS were purchased from Santa Cruz (Santa Cruz, CA, USA). Rabbit polyclonal antibody to eNOS was from Thermo Fisher Scientific (Fremont, CA, USA). N^G -nitro-*L*-arginine methyl ester (*L*-NAME) was purchased from the Beyotime Institute of Biotechnology (Haimen, China).

Cell isolation and culture Human umbilical cords were collected in phosphate-buffered saline (PBS). HUVECs

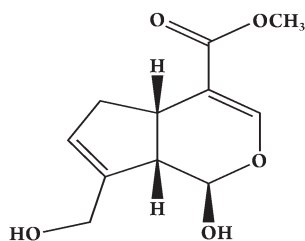


Figure 1. Chemical structure of genipin.

were isolated from freshly obtained human umbilical cords by collagenase digestion of the interior of the umbilical vein^[16–18]. The cell suspension was centrifuged at 1000 r/min for 5 min, and the cell pellet was resuspended in 4 mL of EBM supplemented with 20% fetal calf serum (FCS), 100 U/mL penicillin and 15 μ g/mL ECGS. The cells were plated into 6-well plates and incubated in a humidified incubator at 37 °C under 5 % CO₂.

Cell viability assay The HUVECs were incubated in 96-well plates for 24 h and then treated with various concentrations of genipin or left untreated. After 12-h, 24-h, and 48-h treatments, the cell viability was assessed using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories)^[19,20]. Ten microliters of the CCK-8 solution was added to each well of the plate, and the cells were incubated for 2 h in the incubator (37 °C and 5% CO₂). The absorbance at 450 nm was measured using a microplate reader (Bio-Rad). The cell inhibition rate (I%) was calculated by the following equation: $I\% = (A_{\text{control}} - A_{\text{treated}}) / A_{\text{control}} \times 100\%$.

Measurement of VWF release HUVECs were grown in EBM in 6-well plates until confluent layers were obtained. To measure the effect of genipin on VWF release, the cells were pretreated with various drug concentrations for different lengths of time. The cells were washed with PBS three times and then stimulated with 1 U/mL thrombin for 30 min in EBM medium without FCS. The media were collected and centrifuged at 10 000 \times g for 5 min to remove cellular debris. The VWF concentration in the cell supernatants was measured using a VWF ELISA Kit (USCN Life Science and Technology, Missouri City, TX, USA) according to the operating instructions^[21]. Results are expressed in ng/mL. The samples were assayed in triplicate.

Analysis of P-selectin translocation on the cell surface P-selectin translocation on the cell surface was measured using cell surface ELISA as previously described^[22]. Briefly, HUVECs were cultured in 24-well plates until confluence and then pretreated with various concentrations of genipin for different lengths of time. After stimulation with thrombin or vehicle for 15 min, the cells were fixed for 10 min with 1% paraformaldehyde at room temperature. After three washes with PBS, the fixed cells were probed with goat anti-human P-selectin polyclonal antibody for 1 h and then incubated with horseradish peroxidase-conjugated donkey anti-goat IgG for 1 h. After the cells were washed three times with PBS, TMB was added for 30 min, and then 1 mol/L H₂SO₄ was added to stop the reaction. The absorbance at 450 nm was read in a microplate reader. All assays were done with triplicate samples.

Determination of bleeding times in mice Tail bleed-

ing times in mice were measured as previously described^[23]. Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital, and 5 mm of the distal tip of the tail was amputated. The bleeding end of the tail was blotted with filter paper every 5 s until the paper was no longer stained. If the mice bled continuously for more than 20 min, the experiment was stopped and the bleeding time was recorded as 20 min.

Western blot analysis Cells were lysed with ice-cold lysis buffer. Cell lysates were centrifuged at 10 000 r/min for 5 min at 4 °C, and the supernatants were collected. Protein concentrations in the supernatants were measured using the BCA protein assay kit (Kangchen Bio-tech Inc, Shanghai, China). Then 50 µg protein samples were separated on 10% resolving/4% stacking Tris-HCl gels. Separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA in 1×Tris Buffered Saline, 0.1% TWEEN 20 (TBST) for 1 h at room temperature. Blocked membranes were immunoblotted with antibodies to phospho-eNOS and eNOS overnight at 4 °C. The membranes were then washed and probed with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Chemiluminescence detection was performed with a chemiluminescence detection kit (Kangchen Bio-tech Inc, Shanghai, China) according to the manufacturer's instructions.

Determination of the NO concentration in cell supernatants NO was measured in the cell supernatants as nitrite using a NO Colorimetric Assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol^[17]. Briefly, a standard curve was prepared with standard nitrite solutions in EBM medium, covering a concentration range of 5 to 300 µmol/L. The standard solutions or cell supernatants were reacted with nitrate reductase for 30 min in a 96-well plate, and then Griess reagent I and Griess reagent II were added. After a 15-min incubation at room temperature, the absorbance at 540 nm was read in a microplate reader. The samples were assayed in triplicate.

Data analysis and statistics Data are expressed as the mean±SEM or mean±SD. Variation between groups was analyzed using a one-way ANOVA, which was followed by Student-Newman-Keuls or Dunnett's T3 procedures when the assumption of equal variances did not hold. Two-tailed *P* values <0.05 were considered statistically significant. Statistical analyses were conducted with SPSS 13.0.

Results

Genipin inhibits thrombin-induced VWF exocytosis

from HUVECs To explore the effect of genipin on endothelial cell exocytosis, we first investigated thrombin-induced VWF exocytosis from HUVECs. We pretreated HUVECs with increasing concentrations of genipin for various lengths of time, stimulated the cells with 1 U/mL thrombin for 30 min and then measured the amount of VWF released from the HUVECs. Genipin blocked the release of VWF in a dose-dependent manner, with a maximal effect achieved at 8 µg/mL (Figure 2A). This significant inhibition caused by genipin was seen within 1 h after the start of pretreatment and increased in a time-dependent manner (Figure 2B).

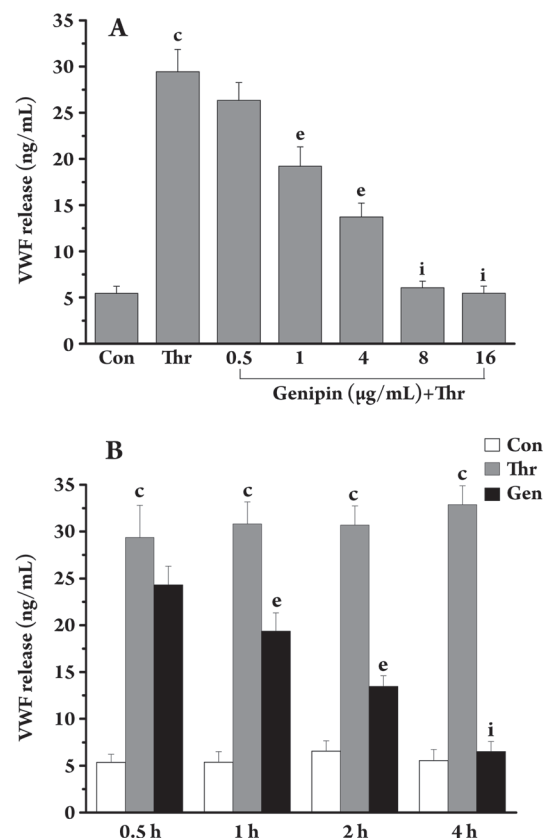


Figure 2. Effect of genipin on thrombin-induced exocytosis of VWF from HUVECs. HUVECs were pretreated with the indicated concentration of genipin for various times or left untreated and then stimulated with 1 U/mL thrombin for 30 min. The VWF concentration in the cell supernatants was measured using a VWF ELISA Kit. (A) Genipin inhibited VWF release in a concentration-dependent manner. (B) Genipin inhibited VWF release in a time-dependent manner. Data are expressed as the mean±SEM of 3 independent experiments. ^c*P*<0.01 vs control (Con); ^e*P*<0.05 vs thrombin (Thr); ⁱ*P*<0.01 vs Thr.

Genipin inhibits thrombin-induced P-selectin translocation from HUVECs To confirm that genipin inhibits endothelial exocytosis, we also determined the effect of geni-

pin on translocation of P-selectin, which is stored in the WPB along with VWF. HUVECs were pretreated with increasing concentrations of genipin for various amounts of time and then stimulated with 1 U/mL thrombin for 15 min. The translocation of P-selectin to the surface of HUVECs was measured using a cell surface ELISA. Thrombin (1 U/mL) caused a rapid increase in P-selectin translocation to the cell surface in control cells. However, genipin inhibited thrombin-induced P-selectin translocation in a dose- and time-dependent manner (Figures 3A and 3B, respectively).

Effect of genipin on HUVEC viability We next examined the effect of genipin on HUVEC viability to determine whether the inhibitory effect of genipin on endothelial exocytosis is related to the cytotoxicity induced by this compound. HUVECs were treated with genipin at doses of

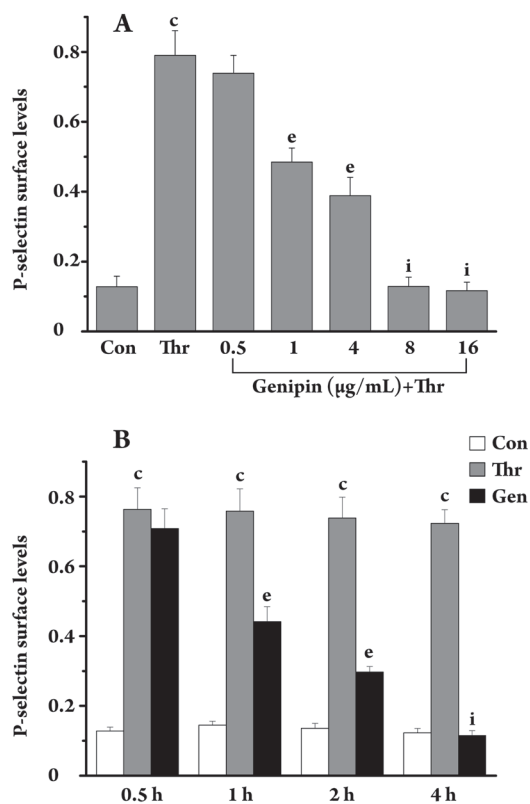


Figure 3. Effect of genipin on thrombin-induced translocation of P-selectin from HUVECs. HUVECs were pretreated with the indicated concentrations of genipin for various times or left untreated and then stimulated with 1 U/mL thrombin for 15 min. P-selectin translocation on HUVEC surfaces was measured using a cell surface ELISA. (A) Genipin inhibited P-selectin translocation in a concentration-dependent manner. (B) Genipin inhibited P-selectin translocation in a time-dependent manner. Data are expressed as the mean±SEM of 3 independent experiments. ^c $P < 0.01$ vs control (Con); ^e $P < 0.05$ vs thrombin (Thr); ⁱ $P < 0.01$ vs Thr.

0.5, 1, 4, 8, and 16 µg/mL for 12, 24, and 48 h. The results showed that genipin treatment had no effect on HUVEC viability (Table 1).

Genipin prolongs the bleeding time in mice Because platelet adherence to the vessel wall is mediated by VWF and P-selectin, we predicted that genipin-induced inhibition of endothelial exocytosis would decrease platelet adherence and prolong the bleeding time. Thus, we measured the effect of genipin on bleeding time in mice to obtain functional evidence that genipin inhibits HUVEC exocytosis *in vivo*. Anesthetized mice were injected intravenously with various concentrations of genipin or PBS. At various times after drug injection, the distal tip of tail was amputated, and the bleeding time was measured. Treatment with PBS had no effect on the bleeding time, with the mice having an average bleeding time of approximately 6 min (Figure 4A). In contrast, genipin dramatically prolonged the bleeding time in a dose-dependent manner (Figure 4A). At doses of 5, 12.5, 25, and 50 mg/kg, the bleeding times were 8, 10, 16, and 18 min,

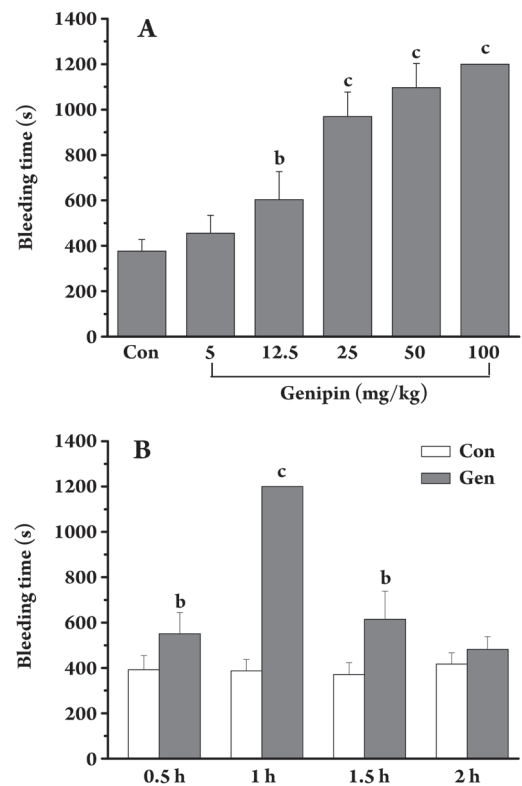


Figure 4. Effect of genipin on the bleeding time in mice. Mice were injected intravenously with different concentrations of genipin. At various times after injection, the bleeding time was measured. (A) Genipin prolonged the bleeding time in mice in a dose-dependent manner. (B) Genipin prolonged the bleeding time. Data are expressed as the mean±SD of 8 mice. ^b $P < 0.05$, ^c $P < 0.01$ vs control (Con).

Table 1. Effect of genipin on HUVEC viability. The HUVECs were incubated in 96-well plates for 24 h, and then treated with the indicated concentration of genipin. After 12 h, 24 h, and 48 h of treatment, the cell viability assessment was performed using Cell Counting Kit-8. Data are expressed as mean±SEM of 3 independent experiments.

Concentration ($\mu\text{g/mL}$)	Cell viability ($OD_{450\text{nm}}$)		
	12 h	24 h	48 h
0	0.73±0.25	0.96±0.37	1.23±0.32
0.5	0.81±0.26	0.98±0.35	1.25±0.39
1	0.87±0.27	0.91±0.32	1.31±0.37
4	0.74±0.33	0.93±0.36	1.38±0.39
8	0.77±0.21	0.86±0.32	1.35±0.31
16	0.83±0.38	0.93±0.35	1.33±0.36

respectively. When the dose of the drug was increased to 100 mg/kg, the bleeding time was longer than 20 min. The maximum genipin-induced bleeding time occurred when the amputation was done 1 h after the drug treatment. The bleeding times returned to normal levels 1.5 h after genipin

treatment.

NO mediates genipin-induced inhibition of HUVEC exocytosis Because genipin has been reported to induce the activation of neuronal NO synthase (nNOS)^[9] and because NO has been shown to inhibit exocytosis of WPBs^[9], we investigated the effect of genipin on the activation of endothelial NOS (eNOS) in order to explore the mechanisms responsible for the inhibitory effects of genipin. HUVECs were treated with different concentrations of genipin or with a control solution for various times. Cell lysates were immunoblotted for phospho-eNOS (Ser-1177) or eNOS. Genipin increased the phosphorylation level of eNOS in a dose- and time-dependent manner (Figures 5A and 5B, respectively), but had no effect on the total amount of eNOS present within 2 h after drug treatment (Figure 5). These results demonstrated that genipin stimulated eNOS phosphorylation and activated eNOS.

To confirm that genipin-induced eNOS activation promotes NO synthesis, we next examined the ability of genipin

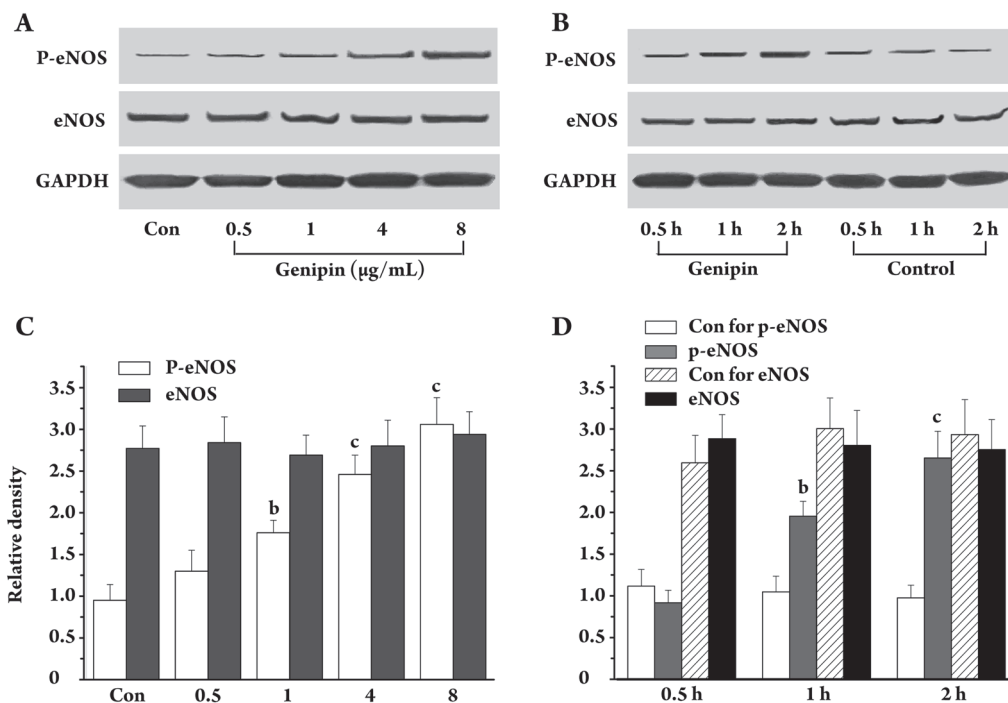


Figure 5. Effect of genipin on eNOS-ser (1177) phosphorylation and total eNOS in HUVECs. HUVECs were treated with the indicated concentrations of genipin for the indicated times or left untreated. Total protein samples were electrophoresed and separated on a 10% SDS-PAGE gel. The levels of phosphorylated eNOS-ser (1177) and total eNOS were detected using Western blot analysis. (A) Representative Western blots of eNOS-ser (1177) phosphorylation and total eNOS for the indicated concentration of genipin. (B) Representative Western blots of eNOS-ser (1177) phosphorylation and total eNOS for the indicated lengths of genipin treatment. (C) Semiquantitative analysis of proteins showed that genipin increased eNOS-ser (1177) phosphorylation in a concentration-dependent manner, but had no effect on the total amount of eNOS in HUVECs. (D) Semiquantitative analysis of proteins showed that genipin increased eNOS-ser (1177) phosphorylation in a time-dependent manner, but had no effect on the total amount of eNOS in HUVECs. Data are expressed as the mean±SEM of 3 independent experiments. ^b $P < 0.05$, ^c $P < 0.01$ vs control (Con).

to stimulate NO production. We treated HUVECs with various genipin concentrations for different lengths of time and then measured the NO level in the cell supernatants. Our results showed that genipin activated HUVEC synthesis of NO in a dose- and time-dependent manner (Figures 6A and 6B, respectively).

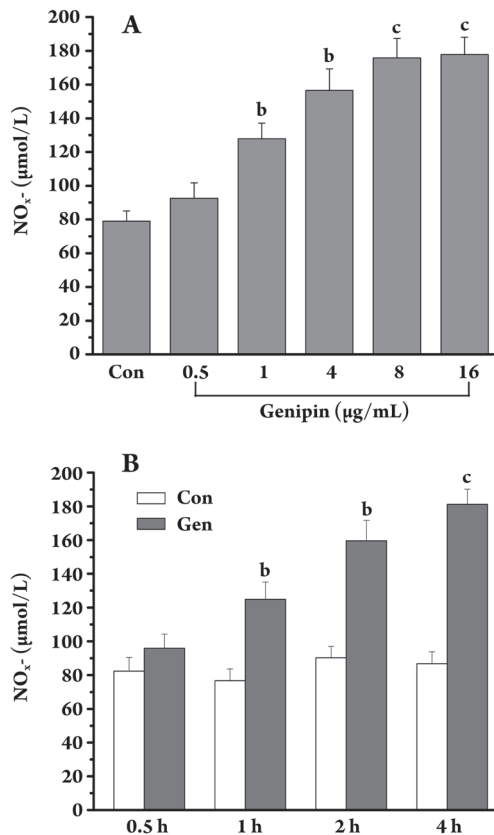


Figure 6. Effect of genipin on nitric oxide production in HUVECs. HUVECs were treated with various concentrations of genipin at different times, and then the NO level was measured in the cell supernatants. (A) Genipin increased NO production in a concentration-dependent manner. (B) Genipin increased NO production in a time-dependent manner. Data are expressed as the mean±SEM of 3 independent experiments. ^b*P*<0.05, ^c*P*<0.01 vs control (Con).

In order to determine whether genipin-induced NO production is involved in the inhibition of exocytosis observed when cells are treated with this compound, we examined the effect of *L*-NAME, an inhibitor of NOS, on genipin-induced inhibition of exocytosis. HUVECs were pretreated with various concentrations of *L*-NAME for 1 h, and then genipin was added for 2 h. The cells were stimulated with thrombin (1 U/mL), and the VWF level in the supernatants

was measured. Our results showed that *L*-NAME reversed the inhibitory effects of genipin on endothelial exocytosis (Figure 7), suggesting that genipin-induced NO production was involved in the genipin-induced inhibition of endothelial exocytosis.

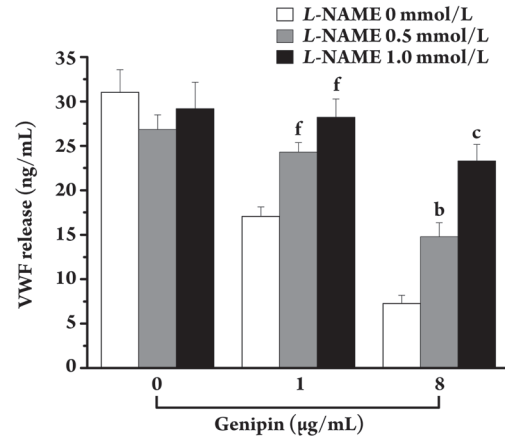


Figure 7. Effect of *L*-NAME on genipin-induced inhibition of VWF exocytosis from HUVECs. HUVECs were pretreated with the indicated concentrations of *L*-NAME for 1 h and then the indicated concentrations of genipin were added for 2 h. The cells were stimulated with 1 U/mL thrombin for 30 min. The VWF concentration in the cell supernatants was measured using a VWF ELISA Kit. Data are expressed as the mean±SEM of 3 independent experiments. ^b*P*<0.05, ^c*P*<0.01 vs *L*-NAME (0 mmol/L) and genipin (8 µg/mL). ^f*P*<0.01 vs *L*-NAME (0 mmol/L) and genipin (1 µg/mL).

Discussion

The main finding of our study is that genipin, the aglycon of geniposide, inhibits endothelial exocytosis in HUVECs. By blocking endothelial exocytosis, genipin prolongs the bleeding time in mice. The mechanism by which this compound inhibits exocytosis may be related to its eNOS activation and stimulation of NO production. Our findings demonstrate that inhibition of exocytosis is a novel anti-inflammatory mechanism of genipin.

WPB exocytosis leads to VWF release and P-selectin translocation. P-selectin is a key leukocyte adhesion molecule that mediates leukocyte adherence to endothelial cells and triggers vascular inflammation^[24]. VWF is a protein that is essential for platelet adhesion and aggregation^[25]. In our study, genipin inhibited endothelial exocytosis from HUVECs. Thus, vascular endothelial cells treated with genipin would translocate less P-selectin and release less VWF. Less P-selectin on the surface of endothelial cells would decrease leukocyte adhesion to the vessel wall^[24].

Less VWF released into the blood would decrease platelet adhesion to the vessel wall^[25]. The collective inhibition of exocytosis of VWF and P-selectin induced by genipin could lead to decreased adhesion of platelets and leukocytes to the vessel wall, limit vascular inflammation and prolong bleeding time. On the other hand, genipin-induced inhibition of platelet aggregation may also be involved in the increased bleeding time caused by this compound^[24]. In addition, we need to point out that an increase in the bleeding time during all genipin treatments should be correlated with VWF release and P-selectin translocation. In our HUVEC study, genipin-induced inhibition of VWF release and P-selectin translocation lasted 4 h or more, whereas in the mouse bleeding study, a single dose of genipin injection prolonged the bleeding time for only about 1 h. The reason for this is that genipin is metabolized rapidly, and the parent form of genipin was not detectable after 60 min when the drug was given intravenously^[26].

How does genipin inhibit endothelial exocytosis? Our results showed that genipin activated eNOS phosphorylation, promoted enzyme activation and increased NO production. An inhibitor of NOS, *L*-NAME, could also reverse the inhibitory effects of genipin on endothelial exocytosis. These results suggest that genipin inhibits endothelial exocytosis by increasing NO production. It has been reported that NO suppresses vascular inflammation and thrombosis by inhibiting WPB exocytosis^[27, 28]. NO inhibits exocytosis by regulating the activity of *N*-ethylmaleimide-sensitive factor (NSF), a critical protein that mediates exocytosis^[27, 28]. However, it should be noted that genipin has a variety of effects on the different NOS isoforms. For example, genipin inhibits NO production and the expression of inducible NOS (iNOS) through the inhibition of nuclear factor κ B (NF- κ B)^[9], but exerts its neurotrophic effects through the activation of neuronal NO synthase (nNOS)^[15]. In fact, another small molecule inhibitor of exocytosis, epigallocatechin gallate, also had different effects on different NOS isoforms, with some studies demonstrating increased iNOS expression and others showing decreased nNOS expression^[29].

It is not clear how genipin acts on eNOS. A genipin target molecule, with a molecular weight of about 170 kDa, has been detected in the cytosolic fraction of rat brain cortex and rat pheochromocytoma PC12h cells^[30]. This target molecule was able to react with anti-neuronal nNOS antiserum, suggesting that nNOS is the target molecule for the neurotogenic action of genipin. Genipin may directly bind to and activate nNOS, thereby causing neurotrophic factor-like activity^[30]. A more recent study has shown that genipin has structural and electronic properties that markedly resemble

those of tetrahydrobiopterin (H4B), an essential cofactor in nNOS^[31]. Further study is needed to determine whether genipin acts on eNOS in the same way as it acts on nNOS.

In conclusion, excessive endothelial exocytosis may contribute to the pathophysiology of inflammation and thrombosis. Genipin, a novel inhibitor of endothelial exocytosis found in this study, may target acute inflammatory events and suppress vascular and endothelial cell inflammatory activation. This compound may represent a new treatment for some types of inflammation and/or thrombosis in which excess endothelial exocytosis plays a pathophysiological role.

Acknowledgements

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Author contribution

Guang-fa WANG, Shao-yu WU, Jia-jie ZHANG and Shu-guang WU designed the research; Guang-fa WANG, Shao-yu WU, Jin-jun RAO, Wei XU and Jian-xin PANG performed the research and analyzed the data; Lin LÜ and Zhong-qiu LIU contributed new analytical reagents and tools; and Guang-fa WANG, Shao-yu WU, Jia-jie ZHANG and Shu-guang WU wrote the paper.

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