

Full-length article

Honokiol inhibits arterial thrombosis through endothelial cell protection and stimulation of prostacyclin

He HU¹, Xiao-xue ZHANG¹, Yin-ye WANG^{1,2}, Shi-zhong CHEN³¹Department of Molecular and Cellular Pharmacology, Beijing University, Beijing 100083, China; ²Department of Natural Medicinal Chemistry, School of Pharmaceutical Sciences, Peking University, Beijing 100083, China

Key words

honokiol; platelet aggregation; thrombosis; cultured cells; endothelium; prostacyclin; nitric oxide

²Correspondence to Prof Yin-ye WANG.

Phn 86-10-8280-2652.

Fax 86-10-6201-5584.

E-mail wangyinye@bjmu.edu.cn

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Abstract

Aim: To study the effect of honokiol on arterial thrombosis and endothelial cells. **Methods:** Rabbit platelet aggregation was performed with Born's turbid method. Thrombosis was produced by the endothelial injury stimulated with electric current. Rat aortic endothelial cells (RAEC) were cultured and cell viability was assessed using the MTT assay. Nitric oxide (NO) concentrations in serum-free media of RAEC were determined using the kinetic cadmium-reduction method. The stable metabolite prostacyclin was measured in serum-free media of RAEC by radioimmunoassay. **Results:** Honokiol (37.6–376 $\mu\text{mol/L}$) decreased rabbit platelet aggregation *in vitro* in a concentration-dependent manner, while intravenously injection of honokiol (0.12–12 $\mu\text{g/kg}$) significantly inhibited rabbit platelet aggregation induced by collagen *ex vivo*. In the electrical current-stimulated carotid thrombosis model in rats, honokiol (5–50 $\mu\text{g/kg}$, iv) prolonged the thrombus occlusion time in a dose-dependent manner. *In vitro* honokiol (0.376–37.6 $\mu\text{mol/L}$) effectively protected cultured RAEC against oxidized low density lipoprotein (ox-LDL) injury, and significantly increased 6-keto-PGF_{1 α} (the stable metabolite of prostacyclin) in serum-free media of RAEC. Honokiol also increased NO level in RAEC serum-free medium at a lower concentration range (0.0376–0.376 $\mu\text{mol/L}$), but honokiol 3.76 $\mu\text{mol/L}$ decreased NO level. **Conclusion:** Honokiol is a potent arterial thrombosis inhibitor. Endothelial cell protection and the stimulation of prostacyclin release may be its main anti-thrombosis mechanism. Stimulation of NO release in endothelial cells may play a role, but it is not a key factor.

Introduction

Platelet activation and aggregation play essential roles in thromboembolic disorders^[1]. When platelets are activated they adhere to the injured blood vessel walls. This results in the formation of an occlusive thrombus in the lumen of the vessel^[2,3]. These thrombi are the source of many thromboembolic cerebral vascular diseases, including strokes^[4].

Honokiol is the main biphenyl neolignan isolated from Hou pu, the cortex of *Magnolia officinalis* (Magnoliaceae), which has been used for treatment of acute enteritis, bacterial or amebic diarrhea, chronic gastritis, etc, in traditional Chinese medicine. The pharmacological effects of honokiol

included inhibition of platelet aggregation by blocking thromboxane A₂ (TXA₂) generation and intracellular calcium mobilization^[5], protection of the myocardium against ischemic injury^[6], and suppression of ventricular arrhythmia^[7]. Recently honokiol was found to protect rat brain from focal cerebral ischemia–reperfusion injury by inhibiting neutrophil infiltration and reactive oxygen species production^[8]. However, the effects of honokiol on thrombosis and its influence on endothelial cells have not yet been reported. The aim of this study was to investigate the effects of honokiol on arterial thrombosis and endothelial cells to explore its potential mechanism of action.

Materials and methods

Chemicals and reagents Honokiol injection was prepared by the Department of Natural Medicinal Chemistry, School of Pharmaceutical Sciences, Peking University (Beijing, China). This water-soluble preparation of honokiol contains polyvinyl and other auxiliary materials. Before use it was diluted with normal saline to different concentrations for intravenous injection (0.5 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$ for rats; 0.12 $\mu\text{g/mL}$, 1.2 $\mu\text{g/mL}$, 12 $\mu\text{g/mL}$ for rabbits; concentrations vary with surface area of the animals). Acetylsalicylic acid (ASA) was the product of Astra (Wuxi, China) and was dissolved in normal saline by sonication before use. Collagen was purchased from Sigma (St Louis, MO, USA) and dissolved in normal saline before use. Oxidized low density lipoprotein (ox-LDL) was the product of Beijing Union Sanyou Science and Technology Development Co (Beijing, China). 6-keto-PGF_{1 α} [the stable metabolite of prostacyclin (PGI₂)] immunoassay kits and nitric oxide (NO)/nitrate assay commercial kits were products of the Radioimmunity Institute of People Liberation Army General Hospital (Beijing, China). α -Tocopherol (VE) was the product of Beijing Double-Crane Pharmaceutical Co (Beijing, China). MTT was the product of Sigma. The control was normal saline plus defined proportion of auxiliary materials.

Experimental animals Male Sprague-Dawley rats and male New Zealand rabbits were obtained from the Experimental Animal Center of Peking University. The experimental procedures were approved by the Local Committee on Animal Care and Use.

Platelet aggregation study *in vitro* Blood samples were obtained from the auditory arteries of Male New Zealand rabbits weighing 3.0–4.0 kg into a syringe containing a 1:10 volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation of blood samples at 120 \times g and 850 \times g, respectively, for 15 min. The platelet count was adjusted to 1.8 \times 10⁹ platelets/mL with PPP. Platelet aggregation was measured using the 490 optical aggregometer (Chrono-log Co. Harvertown, PA USA) as described by Born^[9]. Collagen was used as an inducer with a final concentration of 40–50 $\mu\text{g/mL}$.

Platelet aggregation study *ex vivo* Male rabbits weighing 3.0–3.5 kg were used after overnight fasting. The rabbits were administered intravenously with either control, honokiol 0.12, 1.2, and 12 $\mu\text{g/kg}$ or ASA 1.2 mg/kg. Blood samples were then collected from the auditory arteries of the rabbits into a syringe containing a 1:10 volume of 3.8% sodium citrate at 10 min after administration. PRP and PPP were prepared as above. The platelet count was adjusted to 1.8 \times 10⁹ platelets/

mL with PPP and platelet aggregation was measured with the Chrono-log mode 490 optical aggregometer. Collagen was used as an inducer with a final concentration of 40–50 $\mu\text{g/mL}$.

Carotid thrombosis model in rats induced by electric current Male Sprague-Dawley rats (280–320 g) were anesthetized with urethane (1.5 g/kg, ip) after overnight fasting. The left carotid arteries were then isolated carefully and treated intravenously with either control, honokiol 0.5, 5, 50 $\mu\text{g/kg}$ or ASA 5 mg/kg. Carotid thrombus formation was induced using the modified Hladovec method^[10] on an Electric Thrombosis Stimulator (BT87-3, Baotou Medical College, Baotou, China) by delivering a current of 3 mA for 3 min at 10 min after treatment. Occlusion time (OT) of the arteries was measured through a timer linked to the temperature sensor on the Thrombosis Stimulator.

Culture of rat aortic endothelial cells Endothelial cells were isolated from rat aorta by gentle scraping with vertical ophthalmic forceps. The rat aortic endothelial cells (RAEC) were then grown in T75 polystyrene flasks in the presence of antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin) and subcultured as described by Centra *et al*^[11]. The endothelial cells were allowed to grow undisturbed for several days and thereafter the media were changed every 2 d for a total culturing period of 8–10 d. Cell culture purity (99%) was assessed by staining for factor VIII antigen, as described by Jaffe *et al*^[12] and by visual inspection of their typical morphology. After mechanical disruption of cell monolayers by gentle scraping and triturating, the cells were subcultured. Experiments were carried out using confluent cultures between passages 5 and 7.

Cultured rat aortic endothelial cell injury induced by ox-LDL Rat aortic endothelial cells of passage 5 were seeded at 1 \times 10⁵ cells/mL in 96-well plates and grown to confluence. The cells were treated with control or honokiol (final concentration 0.0376 $\mu\text{mol/L}$, 0.376 $\mu\text{mol/L}$ or 3.76 $\mu\text{mol/L}$) or Vitamin E for 30 min in serum-free medium, and then incubated with ox-LDL (final concentration 50 $\mu\text{g/mL}$) for 24 h as described previously^[13]. The viability of cells was assayed using the MTT assay.

Measurement of 6-keto-PGF_{1 α} levels in serum-free medium of injured rat aortic endothelial cells by ox-LDL The culture medium was removed after RAEC were grown to confluence, and cells were washed twice with phosphate-buffered saline and pre-incubated with the serum-free medium for 30 min. After the cells were treated with control, honokiol 0.376, 3.76, 37.6 $\mu\text{mol/L}$, or VE 100 mg/L at 37 °C for 30 min, the cultures were incubated with ox-LDL (final concentration 50 $\mu\text{g/mL}$) for 24 h to induce injury. Incubations were terminated by placing them in an ice bath. Culture

fluids were saved for the determination of 6-keto-PGF_{1α}, and cells were collected for protein content measurement. 6-keto-PGF_{1α} was determined using immunoassay kits, and protein content was measured using the Bradford method^[14].

Determination of nitric oxide levels in serum-free medium of injured rat aortic endothelial cells by ox-LDL Cells were cultured and treated as described above. The level of NO in culture fluid was determined using a kinetic cadmium-reduction method^[15] with NO/nitrate assay commercial kits.

Statistical analysis The data were expressed as mean±SD and represent data from 5 repeated assays. Statistical evaluation was carried out using the Dunnett *t*-test to compare the differences between treated groups and control groups. *P*<0.05 was considered to be statistically significant.

Results

Effects of honokiol on rabbit platelet aggregation *in vitro* and *ex vivo* As shown in Figure 1, honokiol displayed a concentration-dependent inhibitory effect on platelet aggregation *in vitro*. Honokiol at 0.376 μmol/L did not influence platelet aggregation, but honokiol 37.6 μmol/L decreased the platelet aggregation from 78.0%±5.7% to 13.5%±5.9%, and an almost complete inhibition was observed when 376 μmol/L honokiol was used (from 78.0%±5.7% to 0.6%±1.1%). At 15 min after bolus intravenous administration, honokiol significantly inhibited platelet aggregation induced by collagen. The maximum aggregation rates were 70.0%±7.4% for the control group, 22.9%±17.0 % for the 0.12 μg/kg group, 18.4%±19.8% for the 1.2 μg/kg group and 14.5%±19.0% for the 12 μg/kg group. It was evident that honokiol inhibited collagen-induced platelet aggregation in a dose-dependent manner. ASA, a typical anti-platelet agent, also inhibited collagen-induced platelet aggregation at 1.2 mg/kg under the same conditions (Figure 2). It is evident that honokiol has a

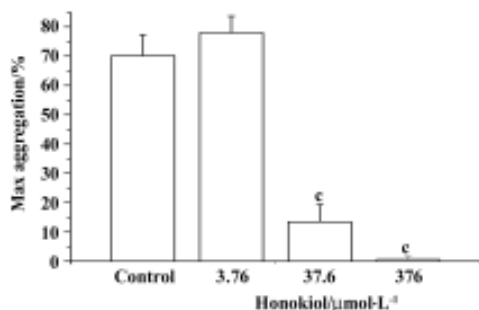


Figure 1. Inhibitory effects of honokiol on collagen-induced platelet aggregation *in vitro*. Platelet aggregation of rabbits was induced by collagen as described by Born. *n*=4 from 4 rabbits. Mean±SD. ^c*P*<0.01 vs control.

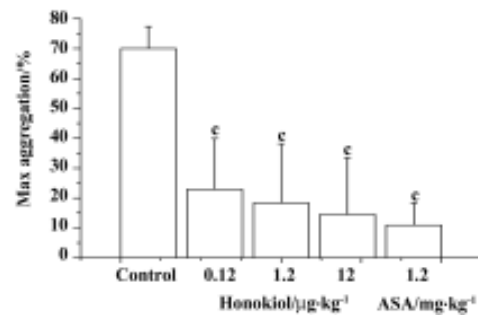


Figure 2. Inhibitory effects of honokiol on collagen-induced rabbit platelet aggregation *ex vivo*. PRP and PPP were prepared from blood samples of rabbits at 15 min after the intravenous administration and platelet aggregation was induced by collagen as described by Born. *n*=6 samples from 6 rabbits. Mean±SD. ^c*P*<0.01 vs control.

potent inhibitory effect on collagen-induced platelet aggregation *ex vivo*; its effective dose is lower than that of ASA.

Effect of honokiol on electrical current-stimulated carotid thrombosis in rats The effects of honokiol on the carotid thrombus model in rats were measured at 10 min after bolus intravenous injection. The OT for the control group was 534 s±66 s, and for the 0.5 μg/kg, 5 μg/kg and 50 μg/kg of honokiol groups the OT were 654 s±204 s, 684 s±186 s and 954 s±282 s, respectively, while the OT for the 5 mg/kg ASA group was 804 s±318 s (Figure 3). It indicated that honokiol dose-dependently inhibited thrombosis induced by endothelium injury *in vivo*. ASA at 5 mg/kg also significantly inhibited thrombosis.

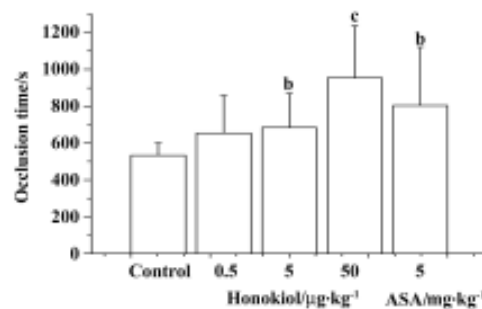


Figure 3. Effects of honokiol on carotid thrombosis. Endothelial injured-thrombus formation in SD rats was stimulated by electric current at 10 min after treatment with control vehicle, honokiol or ASA intravenously. *n*=10 samples from 10 rats. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 vs control.

Effect of honokiol on viability of normal cultured rat aortic endothelial cells Honokiol 0.376–3.76 μmol/L significantly increased cell viability. However, honokiol 37.6 μmol/L and VE 100 mg/L did not influence cell viability. This

suggested that low concentrations of honokiol could stimulate normal endothelial cell proliferation (Figure 4).

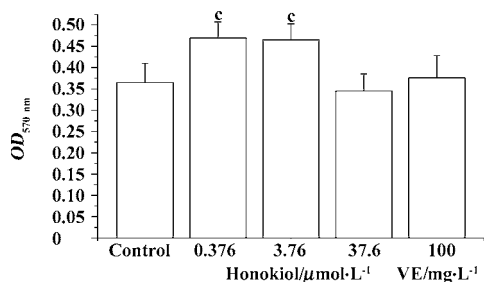


Figure 4. Effect of honokiol on normal rat aortic endothelial cells. The viability of cells was measured using MTT reduction assay. $n=4$ tests. Mean \pm SD. [§] $P<0.01$ vs normal control cells.

Protective effect of honokiol against cultured rat aortic endothelial cell injury induced by ox-LDL The ox-LDL-injured cells showed an obvious decrease in OD values compared with control-treated uninjured cells. Treatment of injured cells with honokiol 0.376–3.76 μmol/L significantly enhanced OD values in a concentration-dependent manner. Honokiol 37.6 μmol/L also increased OD value but no further increase was obtained compared with honokiol 3.76 μmol/L. It indicated that honokiol could protect endothelial cells against ox-LDL injury and increase cell viability. VE 100 mg/L also significantly increased cell viability (Figure 5).

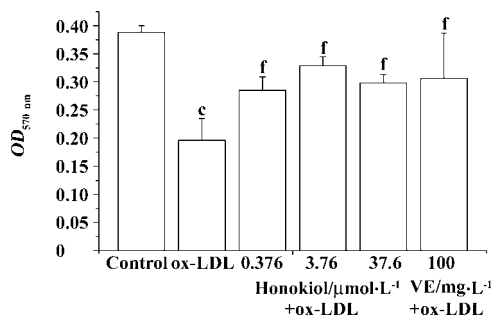


Figure 5. Effect of honokiol on ox-LDL-induced rat aortic endothelial cell injury. The viability of cells was measured using MTT assay. $n=4$ tests. Mean \pm SD. [§] $P<0.01$ vs control cells. ^f $P<0.01$ vs ox-LDL injured cells.

Influence of honokiol on 6-keto-PGF_{1α} concentration in serum-free medium of rat aortic endothelial cells ox-LDL injury significantly decreased the PGI₂ level in serum-free medium. Honokiol 0.376–36.7 μmol/L concentration-dependently increased 6-keto-PGF_{1α} concentration in medium of injured rat aortic endothelial cells, which was significantly

higher than that of injured cells and normal cells. VE 100 mg/L showed a similar result (Figure 6). These results demonstrated that both honokiol and VE were potent PGI₂-release enhancers.

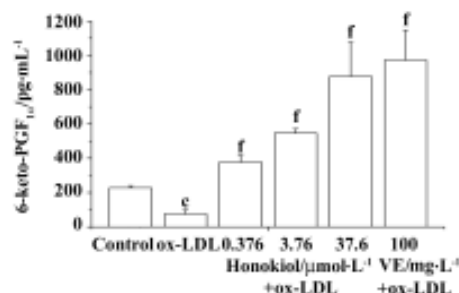


Figure 6. Effect of honokiol on the level of 6-keto-PGF_{1α} in cultured fluid of rat aortic endothelial cells. 6-keto-PGF_{1α} was determined with immunoassay kits. $n=4$ tests. Mean \pm SD. [§] $P<0.01$ vs control. ^f $P<0.01$ vs ox-LDL.

Effect of honokiol on nitric oxide concentration in serum-free medium of rat aortic endothelial cells Honokiol 0.0376–0.376 μmol/L significantly increased NO levels in serum-free medium of rat aortic endothelial cells. However honokiol 3.76 μmol/L did not increase NO level but slightly decreased its level. This result showed that appropriate concentrations of honokiol could promote NO release from rat aortic endothelial cells (Figure 7).

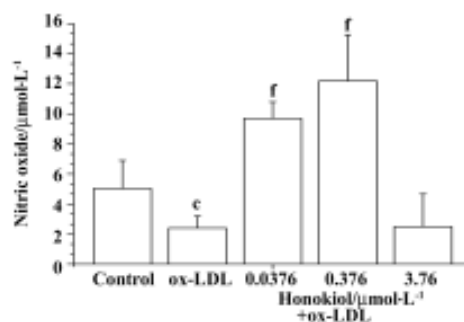


Figure 7. Effect of honokiol on the level of NO in cultured fluid of ox-LDL-injured rat aortic endothelial cells. NO was measured using a kinetic cadmium-reduction method with NO/nitrate assay commercial kits. $n=4$ tests. Mean \pm SD. [§] $P<0.01$ vs control. ^f $P<0.01$ vs ox-LDL.

Discussion

Other authors have demonstrated that honokiol inhibits platelet aggregation in rabbits^[5]. It has protective effects against myocardial ischemia^[6,7] and cerebral infarction^[8]. We

have also found that honokiol can prevent cerebral injury caused by middle cerebral artery occlusion and cerebral ischemia reperfusion injury^[16]. This study demonstrated that honokiol significantly inhibited arterial thrombosis induced by endothelium injury in rats, and the results on platelet aggregation *in vivo* and *in vitro* were basically consistent with those described previously^[5]. We observed that the effective dose of honokiol was much lower than ASA, suggesting that honokiol is a potent platelet aggregation and thrombosis inhibitor. A previous study indicated that the anti-platelet aggregation activity of honokiol was due to its inhibitory effect on TXA₂ formation and intracellular calcium mobilization^[5]. In our preliminary study, honokiol significantly inhibited endothelium-injured thrombosis in rats, and its concentration for inhibiting platelet aggregation *in vitro* was 10 times higher than that for anti-thrombosis. We hypothesize that there is another mechanism mediating its anti-thrombotic effects.

Endothelial cell structure and functional integrity are important in the maintenance of blood vessel walls and circulatory function. They produce and release a variety of vasoactive substances, such as PGI₂ and NO^[17]. PGI₂ is a potent endogenous platelet aggregation inhibitor and produces vasodilatation of all vascular beds studied^[18,19]. We found that honokiol protected cultured RAEC from ox-LDL injury, and it potently increased PGI₂ concentration in cell media in a concentration-dependant manner. The PGI₂ level in cell media treated with honokiol and VE were much higher than that in normal cells, suggesting that both honokiol and VE not only protect cells from injury but also stimulate PGI₂ release from normal RACE. This increase in PGI₂ release may play a crucial role in the anti-thrombotic effect of honokiol. Simultaneously, we have observed that lower concentrations of honokiol 0.376–3.76 μmol/L stimulate normal RACE proliferation, and this effect may be important for maintaining vascular normal state and integrity as well as the concurrence of injured vessels. However, this effect of honokiol needs to be studied further.

At honokiol 37.6 μmol/L, the cell viability is not maximum but PGI₂ level is highest. The reason for this phenomenon is not clear but it is indeed a fact that we confirmed with three separate experiments. We observed that cell shape had a little change at this concentration. Maybe honokiol at this concentration slightly inhibits cell growth or damages cell function. Under this condition the increase of PGI₂ may be a response of cell to injurious stimulation^[20]. This fact suggested that ideal effective concentration of honokiol should be less than 37.6 μmol/L.

According to Teng *et al*, honokiol inhibited TXA₂ forma-

tion in platelets^[5]. This may be due to inhibition of enzymes involved in TXA₂ synthesis, including cyclooxygenase (COX) and TXA₂ synthase in platelets. But our findings suggested that honokiol stimulated PGI₂ release from endothelial cells. A possible explanation for this is that honokiol inhibits TXA₂ synthase in platelets but activates PGI₂ synthase in endothelial cells. However, definitive evidence needs to be provided.

The continuous release of NO from the endothelium has an important role in blood flow modulation. NO may also modulate interactions between inflammatory cells and the endothelium. It reduces platelet, monocyte, macrophages, and neutrophils adhesion to endothelial cells and inhibits platelet aggregation^[21–25]. This study showed that honokiol 0.0376–0.376 μmol/L stimulated NO release, but honokiol 3.76 μmol/L did not and caused a slight decrease in NO level. The effective concentration range of honokiol in stimulating NO release is 10 times lower than that in increasing cell viability. Thereby NO release may not play an important role in the anti-thrombotic effect of honokiol. Accordingly, protection of endothelial cells and stimulation of PGI₂ release from endothelial cells may be the main mechanism by which honokiol inhibited thrombosis, apart from its inhibitory effect on platelet arachidonic acid pathway as described by Teng *et al*^[5]. However, honokiol and VE showed similar effects in cell protection and PGI₂ release under present condition. Whether these effects are the results of their anti-oxidative properties or they themselves have a stimulatory effect on PGI₂ release is not clear yet. In addition, the results of this study cannot explain whether honokiol stimulates PGI₂ generation in endothelial cells. The limitation of this study is that it did not supply direct evidence of the importance of PGI₂ on anti-thrombosis caused by honokiol.

In conclusion, this study showed that honokiol is a potent arterial thrombosis inhibitor. Protection of endothelial cells and stimulation of PGI₂ release may be the main mechanism. NO release from endothelial cells may play some roles but is not a key factor. The detailed mechanism by which honokiol stimulates PGI₂ release and promotes endothelial cell proliferation requires further study.

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