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Honokiol up-regulates prostacyclin synthease protein expression and inhibits endothelial cell apoptosis

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Abstract

Honokiol is a bioactive compound extracted from the Chinese medicinal herb *Magnolia officinalis*. We recently demonstrated that honokiol inhibited arterial thrombosis through stimulation of prostacyclin (PGI_2) generation and endothelial cell protection. The current study is designed to investigate its mechanism of stimulation of PGI_2 generation and cell protection. 6-keto-PGF1α, the stable metabolite of PGI_2, in the media of rat aortic endothelial cells was measured with radioimmunoassay kits. Indomethacin, an inhibitor of cyclooxygenase (COX) and tranylcypromine, a prostacyclin synthease inhibitor were used to ascertain the target enzyme affected by honokiol. Prostacyclin synthease protein levels in endothelial cells were determined by Western blot analysis using an anti-PGI_2 synthease rabbit polyclonal antibody. Flow cytometry was used to quantify the apoptotic cells and spectrophotometry was used to test the caspase-3 activity. Honokiol (0.376–37.6 μM) increased the level of 6-keto-PGF1α in the media of normal endothelial cells. It counteracted the inhibitory effect of tranylcypromine on the PGI_2 generation, but did not influence the effect of indomethacin; evidently, honokiol up-regulated the expression of prostacyclin synthease in the endothelial cells. These effects showed perfect concentration-dependent behavior. In addition, at lower concentration (0.376–3.76 μM), honokiol significantly decreased the percentage of apoptotic endothelial cells induced by oxidized low-density lipoprotein (ox-LDL) and significantly lowered the activity of caspase-3 stimulated by ox-LDL. A high dose of honokiol (37.6 μM), however, failed to influence either of them. In conclusion, honokiol augments PGI_2 generation in normal endothelial cells; its effect on PGI_2 generation attributes to up-regulation of prostacyclin synthease expression; its cell protection may be correlated with its inhibition on apoptosis of endothelial cells. These findings have partly revealed the molecular mechanism of honokiol on inhibiting arterial thrombosis.

Keywords: Honokiol; Thrombosis; Rat aortic endothelial cells; Prostacyclin (PGI_2); Prostacyclin synthease; Apoptosis

1. Introduction

Thrombosis is a major cause of cardiovascular and cerebrovascular diseases. Endothelial cells play important roles in controlling thrombosis by synthesis and release of endogenous active substances such as prostacyclin (PGI_2), nitric oxide (NO), tissue-plasminogen, etc. It is these substances that are directly involved in regulating blood flow and vascular stress. Structural and functional integrity of endothelial cells are important in the maintenance of vessel walls and circulatory function. Endothelial dysfunction facilitates a number of unfavorable effects on vasculature, including platelet activation on injured endothelium (Cines et al., 1998), which then leads to an imbalance in endothelium-derived factors and, thereafter, induction of the formation and development of thrombi (Golino et al., 1989; Kishi and Numano, 1989; Watanabe et al., 1997).

Honokiol is a main biphenyl neolignan isolated from traditional Chinese medicine Hou p'u, cortex of *Magnolia officinalis* (*Magnoliaceae*), which has a variety of pharmacological effects such as anti-oxidation (Lo et al., 1994; Chiu et al., 1997), anti-platelet aggregation (Teng et al., 1988), anxiolytic effect (Watanabe et al., 1983), etc. Recently, we demonstrated that honokiol inhibited arterial thrombosis through endothelial cell protection and stimulation of PGI_2 generation (Hu et al., 2005), but the detailed mechanisms of these effects were not clear yet. In current study we explored its action mechanism on the generation of PGI_2 and on cell protection, further explaining honokiol's anti-thrombotic effects.

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2. Materials and methods

2.1. Materials

Honokiol injection was prepared by the Department of Natural Medicinal Chemistry, Peking University. The water-soluble preparation of honokiol contains polyvinyl and other auxiliary materials. RPMI Medium 1640 was the product of Invitrogen Corporation, USA; NBCS (Newborn Calf Serum) was the product of PAA Laboratoies GmbH; and ox-LDL was the product of Beijing Union Sanyou Science & Technology Development Corporation Ltd, China. Rnase, indomethacin, arachidonic acid and tranylcypromine were all products of Sigma, USA; 6-keto-PGF1α immunoassay kits were products of Radioimmunology Institute of People's Liberation Army General Hospital, Beijing; Cell lysis buffer was the product of Beyotime Biotechnology, China; and the vehicle control was normal saline plus definite proportion of auxiliary materials.

2.2. Cell culture

Male Sprague–Dawley rats weighing 180–200 g were anesthetized with an overdose of sodium pentobarbital and the abdominal aortas of rats were rapidly removed and collected in 1640 medium. Surrounding fat and connective tissue were cleaned off, and then aortas were opened longitudinally. Cells from the endothelium were harvested by gently scraping the luminal surface using vertical ophthalmic forceps. The cells were then seeded in T75 polystyrene flasks pre-coated with sterile murine tail collagen in the medium 1640 containing 20% Newborn Calf Serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin, and cultured at 37 °C in a humidified 5% CO_2 atmosphere. Cells were allowed to grow undisturbed for 4 days and thereafter the media were changed every 2 days for a total culturing period of 8 to 10 days. The endothelial cells were identified according to the previous method (Jaffe et al., 1973) and were subcultured as described by others (Centra et al., 1992). Experiments were performed using confluent cultures of passage 5 (Sibel et al., 2001).

2.3. Measurement of 6-keto-$PGF_1\alpha$ in media of normal rat aortic endothelial cells

The rat aortic endothelial cells were seeded into 96-well plates with 1×10^5 cells/ml and cultured until confluence. The media were removed and cells were washed twice with PBS, and pre-incubated for 30 min with the medium 1640 containing 2% Newborn Calf Serum. The cultures were then incubated for 24 h with vehicle, 0.376, 3.76 or 37.6 μM of honokiol respectively. Culture media were saved for the determination of 6-keto-$PGF_1\alpha$, which was determined with immunoassay kits.

2.4. Intervention experiments of inhibitors of cyclooxygenase (COX) and prostacyclin synthease

The rat aortic endothelial cells were seeded into 96-well plates with 1×10^5 cells/ml and cultured until confluence. The media were removed and cells were washed twice with PBS, and then pre-incubated with the medium 1640 containing 2% Newborn Calf Serum for 30 min. After pre-treated with vehicle, 0.376, 3.76 or 37.6 μM of honokiol at 37 °C for 30 min, the cultures were incubated with indomethacin (3 μM) for 24 h (Ottino and Duncan, 1997) to evaluate the influence of honokiol on COX. In the other set of samples, after treated with vehicle, 0.376, 3.76 or 37.6 μM of honokiol at 37 °C for 30 min, the cultures were incubated with the prostacyclin synthease inhibitor tranylcypromine (1 mM) for 24 h to evaluate the influence of honokiol on prostacyclin synthease (Debbie and Mary, 1995). The vehicle controls and inhibitor controls were set up to observe their influences. Arachidonic acid (30 μM) was added to all cultures to supply sufficient substrate. Culture fluids were saved for the determination of PGI_2, which is the final product of COX or prostacyclin synthease. 6-keto-PGF1α in cultured fluid was determined with immunoassay kits.

2.5. Western blot analysis of prostacyclin synthease

The rat aortic endothelial cells were seeded into 6-well plates with 2×10^5 cells/ml and cultured until confluence. Endothelial cells were treated with tumor necrosis factor-α (TNFα) (10 ng/ml), vehicle, 0.376, 3.76 or 37.6 μM of honokiol for 24 h, respectively. After removal of the media, cells were washed twice with ice-cold PBS, then lysed using cell lysis buffer [20 mM Tris PH7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na3VO4, 0.5 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF)]. The lysates were collected by scraping from the plates, and then centrifuged at 10,000 ×*g* at 4 °C for 5 min. Western blot was performed according to the procedure of Towbin et al. (1979). Briefly, proteins (30 μg) were loaded on a 12% of SDS-polyacrylamide gel for electrophoresis, then transferred onto nitrocellulose transfer membranes (Osmonics, USA) at 0.8 mA/cm^2 for 2 h. Membranes were blocked at room temperature for 1 h with blocking solution [5% skimmed milk in Tris-buffered solution plus Tween-20 (TBST): 50 mM Tris–HCl, 150 mM NaCl, pH = 7.5, 0.1% v/v Tween 20]. Membranes were then incubated overnight at 4 °C with anti-PGI_2 synthase rabbit polyclonal antibody at 1:150 (Santa Cruz Biotechnology, USA) in blocking solution. After two 10 min washing in TBST, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit secondary antibody at 1:500 in blocking solution. Detection was performed by enhanced chemiluminescenc (ECL) using a Western Blotting Luminol Reagent (Santa Cruz Biotechnology, USA) according to the manufacturer's instructions. Bands were then quantified by scanning densitometry (THERMAL IMAGING SYSTEM FTI-500, Pharmacia Biotech). Protein concentrations were determined using the Pierce Micro BCA protein assay system (Pierce, Rockford, Illinois, USA). β-actin of rat aortic endothelial cells was used as a housekeeping protein, and was determined following the same procedure as mentioned above, using a specific anti-actin mouse monoclonal antibody (Sigma-Aldrich, Madrid, Spain) at 1:1000 in blocking solution and a horseradish peroxidase-

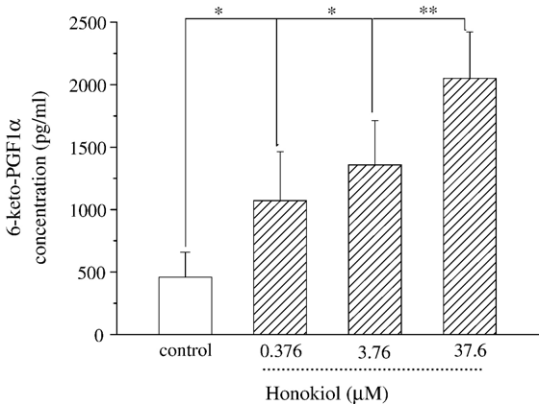

Fig. 1. Effect of honokiol on the level of 6-keto-$PGF_1\alpha$ in media of normal rat aortic endothelial cells. Rat aortic endothelial cells of passage 5 were seeded with 1×10^5 cells/ml in 96-well plates and grown to confluence, then treated with vehicle and honokiol for 24 h, respectively. 6-keto-$PGF_1\alpha$ in culture fluids was determined with immunoassay kits. Each datum was from 6 repeated tests, and expressed as mean±S.D. $^*P<0.05$; $^{**}P<0.01$.

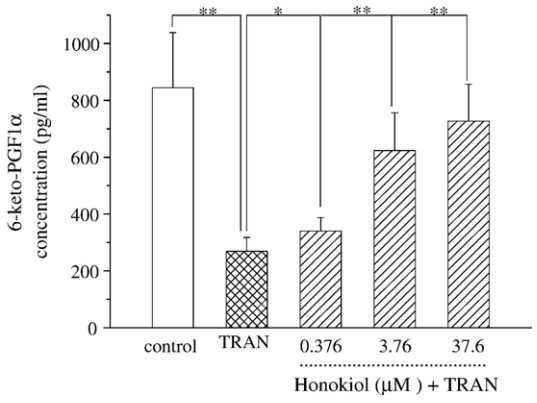

Fig. 3. Influence of honokiol on prostacyclin synthease inhibited by tranylcypromine. Rat aortic endothelial cells of passage 5 were seeded at 1×10^5 cells/ml in 96-well plates and grown to confluence, then were exposed to tranylcypromine (1 mM) for 24 h after treated with vehicle or honokiol for 30 min. 6-keto-$PGF_1\alpha$ in culture fluids was determined with immunoassay kits. Each datum was from 6 repeated tests, and expressed as mean±S.D. $^*P<0.05$, $^{**}P<0.01$. "TRAN" represents tranylcypromine.

conjugated anti-mouse secondary antibody at 1:500 in blocking solution.

2.6. Flow cytometry analysis

The rat aortic endothelial cells were seeded into 60 mm culture dishes with 5×10^5 cells/ml and cultured until confluence. The cells were washed twice with PBS and pre-incubated for 30 min with the medium 1640 containing 2% Newborn Calf Serum. The cells were then treated with vehicle, 0.376, 3.76 or 37.6 μM of honokiol at 37 °C for 30 min, respectively, and incubated with oxidized low-density lipoprotein (ox-LDL) (final concentration was 100 μg/ml) for 24 h to induce cell apoptosis. The degree of apoptotic cells was analyzed by flow cytometry as previously described (Chaouchi et al., 1994). Shortly, pelleted cells were suspended and fixed in 70% cold ethanol overnight at −20 °C after twice washing with PBS. Then the cells were re-suspended in PBS containing RNase A (200 μg/ml), and incubated at 37 °C for 30 min. Propidium iodide PI was added to the cell suspensions to give final concentrations of 100 μg/ml. The fluorescence intensity of

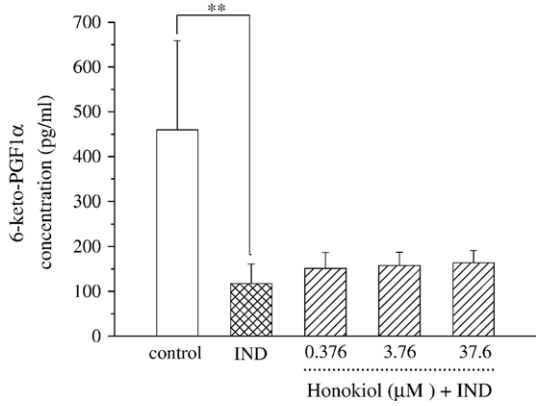

Fig. 2. Influence of honokiol on COX inhibited by indomethecin. Rat aortic endothelial cells passage 5 were seeded at 1×10^5 cells/ml in 96-well plates and grown to confluence, then were exposed to indomethecin (3 μM) for 24 h after treated with vehicle or honokiol for 30 min. 6-keto-$PGF_1\alpha$ in culture fluids was determined with immunoassay kits. Each datum was from 6 repeated tests, and expressed as mean±S.D. $^{**}P<0.01$. "IND" represents indomethecin.

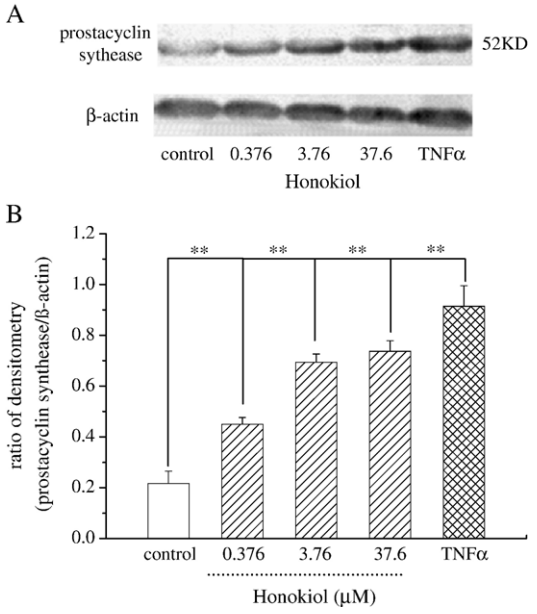

Fig. 4. Effect of honokiol on prostacyclin synthease protein expression in rat aortic endothelial cells. Rat aortic endothelial cells of passage 5 were seeded at 2×10^5 cells/ml in 6-well plates and grown to confluence, then were exposed to vehicle, TNFα (10 ng/ml) or honokiol for 24 h. Then Western blot analysis of cellular proteins from different treated cells was performed. A) The bands of prostacyclin synthease and β-actin were identified by Western blot analysis using an anti-prostacyclin synthease rabbit polyclonal antibody and anti-actin mouse monoclonal antibody, in which β-actin was used as a loading control. Shown was one Western blot result of 3 separate experiments; B) The statistical analysis results of ratio of prostacyclin synthease compared to β-actin. Results were depicted as mean±S.D. $^*P<0.05$, $^{**}P<0.01$.

PI was analyzed with a FACScalibur flow cytometer and CellQuest software.

2.7. Caspase-3 activity assay

The rat aortic endothelial cells were seeded into 24-well plates with 2×10^6 cells/ml and cultured until confluence, and the apoptosis was induced as mentioned above. At the same time, honokiol controls were set up in which cells were treated only with honokiol (0.376, 3.76, and 37.6 μM) without ox-LDL. After induced apoptosis the activity of caspase-3 in cells was measured with spectrophotometry at 405 nm (MICROPLATE READER MODEL 550, BIO-RAD) using a commercially available kit (Calbiochem) according to the manufacturer's instruction, which detects chromophore *p*-nitroanilide after cleavage from the labeled substrate *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide.

2.8. Statistical analysis

The data are expressed as mean±S.D. Statistical evaluation was performed using Dunnet *t*-test to compare the differences between treated groups and control groups. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Influence of honokiol on PGI_2 generation in normal rat aortic endothelial cells

Normal endothelial cells treated with 0.376–37.6 μM of honokiol produced a significant concentration-dependent increase in the level of 6-keto-$PGF_1\alpha$ in media compared with vehicle-treated cells (Fig. 1), it was demonstrated that honokiol augmented the generation of PGI_2 besides its protective effect on endothelial cells thereby increasing PGI_2 generation as our previous presupposition (Hu et al., 2005).

3.2. Influence of honokiol on COX inhibited by indomethacin

In the presence of COX inhibitor the concentration of 6-keto-$PGF_1\alpha$ in the media was measured to evaluate the effect of honokiol on COX. The treatment with indomethacin plus vehicle resulted in a significant decrease on the concentration of 6-keto-$PGF_1\alpha$ in the media. The treatment with indomethacin plus 0.376, 3.76 or 37.6 μM of honokiol failed to change the effect of indomethacin (Fig. 2). The result demonstrated that honokiol did not influence COX in the presence of the inhibitor. In other words, the influence of honokiol on PGI_2 is irrelative with COX.

3.3. Influence of honokiol on prostacyclin synthease inhibited by tranylcypromine

Determining the concentration of 6-keto-PGF1α in endothelial cells media was used to assess the influence of honokiol on prostacyclin synthease. The treatment with tranylcypromine,

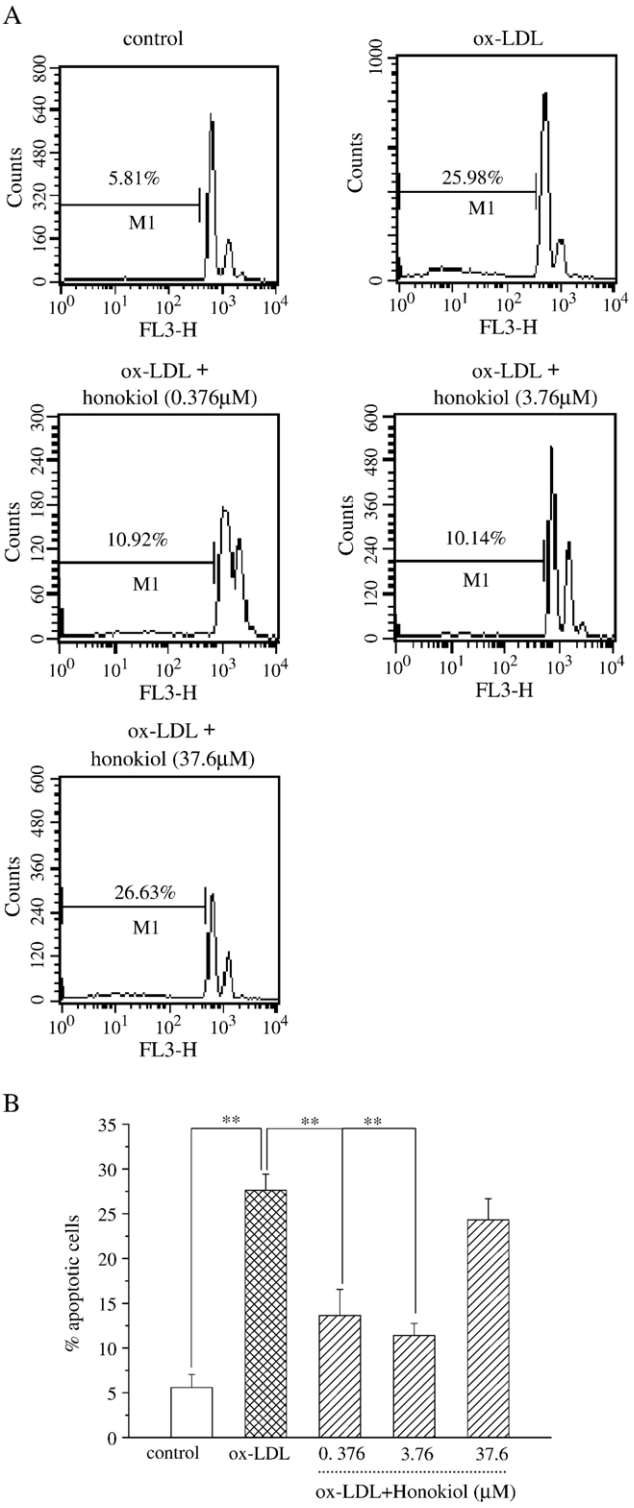

Fig. 5. Effect of honokiol on rat aortic endothelial cells apoptosis induced by ox-LDL. Rat aortic endothelial cells of passage 5 were seeded at 5×10^5 cells/ml in 60 mm culture dishes and grown to confluence. After pre-incubated with vehicle or honokiol for 30 min, cultures were exposed to ox-LDL for 24 h to induce apoptosis; the cells were then collected to determine apoptotic percentages. Apoptotic cells were quantified by FACS analysis of cell nuclei after staining with propidium iodide. Cells containing amounts of DNA found in sub-G1 peak are in region M1, and the ratio of M1 and total region represented the percentage of apoptotic cells. A) Shown was one representative of apoptosis determination from 3 repeated separate experiments. B) The statistical analysis results of percentage of apoptotic cells. Results were depicted as mean±S.D. $^{**}P<0.01$.

a prostacyclin synthease inhibitor, resulted in a significant decrease on the concentration of 6-keto-$PGF_1\alpha$ compared with vehicle. 0.376–37.6 μM of honokiol reversed the influence of prostacyclin synthease inhibitor on 6-keto-$PGF_1\alpha$ with a concentration-dependent behavior (Fig. 3). The results indicate that prostacyclin synthease was the target enzyme affected by honokiol.

3.4. Effect of honokiol on prostacyclin synthease protein expression

We investigated the effect of honokiol on prostacyclin synthease expression with western blot analysis. TNFα was used as a positive control according to other study (Romualdo et al., 1997). The treatment with honokiol or TNFα increased the prostacyclin synthease protein levels compared with vehicle, and the effect of honokiol exhibited a concentration dependent manner (Fig. 4). This result manifested that honokiol stimulates prostacyclin synthease expression in rat aortic endothelial cells; it may be a promoter of prostacyclin synthease expression.

3.5. Effect of honokiol on apoptosis of rat aortic endothelial cells

Flow cytometry was used to quantify the amount of apoptotic cells. The amount of bound dye correlates with the DNA content in a given cell counts, and DNA fragmentation in apoptotic cells translates into fluorescence intensity lower than that of G1 cells (sub-G1 peak) corresponding to apoptotic cells. The percentage of apoptosis for 0.376 and 3.76 μM of honokiol treated cells were significantly lowered compared with vehicle treated cells, but treatment with 37.6 μM of honokiol failed to influence the percentage of apoptosis (Fig. 5), indicating that at appropriate concentrations (0.376–3.76 μM) honokiol inhibits endothelial cell apoptosis, which may be one of its mechanisms for cell protection.

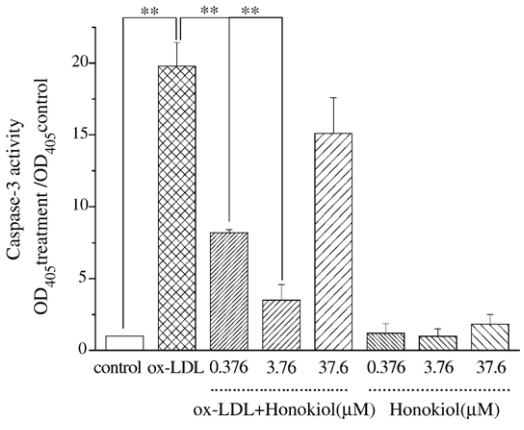

Fig. 6. Effect of honokiol on the caspase-3 activity. Rat aortic endothelial cells of passage 5 were seeded at 2×10^6 cells/ml in 24-well plates and grown to confluence. After pre-incubated with vehicle or honokiol for 30 min, cultures were exposed to ox-LDL for 24 h to induce apoptosis, and in the other cultures, cells were only incubated with honokiol to evaluate honokiol per se effect on apoptosis of endothelial cells. The activity of caspase-3 was measured spectrophotometrically at 405 nm using a commercially available kit. $OD_{405treatment}/OD_{405control}$ was used to express the increase in caspase-3 activity. Results were depicted as mean±S.D. $^{**}P<0.01$.

3.6. Effect of honokiol on caspase-3 activity of apoptotic rat aortic endothelial cells

Caspase-3 activity induced by ox-LDL in the presence or absence of honokiol was analyzed. As shown in Fig. 6, honokiol (0.376–3.76 μM) significantly inhibited the caspase-3 activity induced by ox-LDL, but a high dose of honokiol (37.6 μM) failed to show effect. However, honokiol did not influence the caspase-3 activity of normal rat aortic endothelial cells, which was consistent with our previous finding that honokiol caused no damage to endothelial cells (Hu et al., 2005).

4. Discussion

Honokiol demonstrated platelet aggregation inhibition by inhibiting thromboxane A2 (TXA_2) formation in platelets (Teng et al., 1988). Recently it was found to prevent cerebral injury caused by middle cerebral artery occlusion and cerebral ischemia reperfusion injury, the mechanism for these effects maybe its antioxidant activity (Liou et al., 2003; Chen et al., 2003). More recently it was discovered that honokiol significantly inhibited arterial thrombosis induced by endothelium injury in rats, which may be attributed to its protective effect on endothelial cells and its stimulation of PGI_2 generation (Hu et al., 2005). Therefore, it is presupposed that its protective effect on injured rat aortic endothelial cells may be one of the reasons for the increase in the PGI_2 generation. The viability of cells treated with 37.6 μM of honokiol is not highest, but the PGI_2 level in media is the highest at this concentration (Hu et al., 2005), which leads to the conclusion that there should be other mechanisms than cells protection for PGI_2 level increase. In this study, honokiol (0.376–37.6 μM) significantly and concentration-dependently increased PGI_2 level in normal rat aortic endothelial cells media, which further suggested that honokiol increased PGI_2 production through pathways other than the protection of endothelial cells.

COX and prostacyclin synthease are the two rate-limiting enzymes of PGI_2 synthesis in endothelial cells (Feng et al., 1993; Spisni et al., 1995). Therefore, COX inhibitor indomethacin or prostacyclin synthease inhibitor tranylcypromine were used to explore the effects of honokiol on the two enzymes. The results showed that honokiol still enhanced the PGI_2 level in endothelial cells media in the presence of prostacyclin synthease inhibitor, but did not influence the effect of COX inhibitor, which suggested that the target of honokiol's effect was prostacyclin synthease not COX. The opinion that the target enzyme of honokiol's action was prostacyclin synthease seems disagreed with the idea that honokiol inhibited TXA_2 formation in platelets (Teng et al., 1988). One possible explanation is that honokiol inhibits TXA_2 synthesis in platelets, and augments PGI_2 generation in endothelial cells.

In addition, honokiol up-regulated prostacyclin synthease protein levels in endothelial cells in this study (Fig. 4). Honokiol

was certainly an enhancement factor for PGI_2 generation. Therefore the stimulation of honokiol on PGI_2 production is attributed to the increase of prostacyclin synthease protein expression.

Abnormalities of endothelial cell structure and function may contribute to the occurrence of diseases such as thrombosis and atherosclerosis. There are two mechanisms of cell death: apoptosis and necrosis. ox-LDL can induce apoptosis in endothelial cells (Lin et al., 2004; Zhang et al., 2003). Honokiol protected endothelial cells against ox-LDL injury (Hu et al., 2005) and showed significant anti-oxidation effect (Lo et al., 1994). To further reveal the mechanisms of its cell protection, the effect of honokiol on ox-LDL-induced apoptosis was explored in this study. The results of flow cytometry analysis and caspase-3 activity assay indicated the inhibitory effect of honokiol on the endothelial cell apoptosis was dependent on the concentration. 0.376–3.76 μM of honokiol significantly inhibited cell apoptosis, whereas a higher concentration (37.6 μM) failed to show this effect (Figs. 5 and 6). However, honokiol at all these concentrations (0.376–37.6 μM) did not change the activity of caspase-3 of normal endothelial cell (Fig. 6); suggesting that honokiol did not cause the apoptosis of normal cells at these concentrations.

It was reported that 40 μM of honokiol induced human squamous lung cancer CH27 cells apoptosis (Yang et al., 2002). This indicates that at high concentrations, honokiol may influence cells differently. In our previous study, it was found that the protective effect of honokiol at 37.6 μM on endothelial cells was not more potent than at 3.76 μM (Hu et al., 2005). These manifestations may be due to its maximum effect having been reached at 3.76 μM, indicating that at much higher concentrations (37.6 μM or higher) activity would not increase or decrease. There are various mechanisms of cell protection including inhibiting apoptosis, anti-oxidation and scavenging free radicals, etc. Based on the data of this study, the effect of honokiol was dependent on concentration, at lower concentration the inhibition of apoptosis was the central mechanism for protective effect of honokiol, while at higher concentrations the other factors were important (such as anti-oxidation). In accordance to the results it can be explained that 0.376–3.76 μM of honokiol significantly inhibited cell apoptosis, but higher concentrations (37.6 μM) failed. However, the detailed mechanism of honokiol on cell protection needs to be further explored.

In conclusion, honokiol augments PGI_2 generation in normal endothelial cells; its effect on PGI_2 generation attributes to up-regulation of prostacyclin synthease expression; its cell protection is correlated with its inhibition on apoptosis of endothelial cell. These findings have partly revealed the molecular mechanism of honokiol on inhibiting arterial thrombosis.

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