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Icariin improves eNOS / NO-pathway to prohibit the atherogenesis of apolipoprotein E null mice

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Abstract: Impaired endothelial nitric oxide synthesis (eNOS) / nitric oxide (NO) pathway induce the atherogenesis. The present study examined whether icariin improves the eNOS / NO pathway to prohibit the atherogenesis of apolipoprotein E-null (ApoE^{-/-}) mice. *In vitro*, primary human umbilical vein endothelial cells (HUVECs) were randomly divided into 7 groups: control, vehicle; icariin 10; LPC group; LPC + icariin 1; LPC + icariin 3; LPC + icariin 10. *In vivo*, 80 mice were separated randomly into four groups (n = 20): control, ApoE^{-/-}, ApoE^{-/-} + icariin 10, and ApoE^{-/-} + icariin 30. ApoE^{-/-} mice had significantly more atherosclerosis in the aortic root together with increased aortic ROS production, body weight, plasma triglyceride (TG) and total cholesterol (TC) concentration, decreased aortic eNOS expression, and plasma NO concentration. LPC (10 µg/ml) treatment induced a big decline in NO level in the conditioned medium and eNOS expression, an increase in intracellular reactive oxygen species (ROS) production in HUVECs. Icariin treatment decreased atherogenesis, ROS production, body weight, plasma TG concentration, and plasma TC concentration, increased NO concentration and eNOS expression. These findings suggested icariin could improve eNOS / NO-pathway to prohibit the atherogenesis of apolipoprotein E null mice by restraining oxidative stress.

Keywords : endothelial nitric oxide synthesis; nitric oxide; atherogenesis; icariin

Introduction

Nitric oxide (NO) is a key modulator of vascular disease. It has many intracellular effects that result in endothelial regeneration, vasorelaxation, platelet adhesion, and the constraint of leukocyte chemotaxis. Through the production of NO, lots of usually

used vasculoprotective agents have their therapeutic actions, which show favorable effects on atherosclerosis. Endothelial nitric oxide synthase (eNOS) could catalyze NO production (Napoli et al. 2006). Oxidative stress facilitates atherogenesis. Previous investigations have reported that elevated concentrations of ROS decrease the amount of bioactive NO (Förstermann 2010). Oxidative stress also leads to eNOS uncoupling (Li et al. 2014). Therefore, the eNOS / NO pathway may be a pharmacologic target for improving the atherosclerosis.

Also known as yin yang huo, fairy wings, barrenwort, bishop's hat, horny goat weed, epimedium is a genus of flowering plants in the family berberidaceae. Icariin is the prenyl acetylation of kaempferide 3, 7-O-diglucoside, which can be found in several plants in the berberidaceae family. Extracts from these plants are known for supporting healthy sexual function and producing aphrodisiac effects. There is evidence to suggest that icariin weakens the prothrombotic state in atherosclerotic rabbits (Zhang et al. 2013), but the mechanism responsible for its inhibition on atherogenesis is not yet outright described. As has been mentioned above, oxidative stress impairs eNOS / NO pathway. Previous investigations have shown that icariin has potent antioxidant activity (Pan et al. 2005). Based on its antioxidant properties, we postulated that icariin could modulate eNOS / NO pathway to improve the atherosclerosis by inhibiting oxidative stress.

Therefore, we tested if icariin improves the eNOS / NO pathway to prohibit the atherogenesis of apolipoprotein E- null ($\text{ApoE}^{-/-}$) mice in the present study.

Materials and methods

Reagents

Antibody, lophosphatidylcholine (LPC), and icariin (purity: 98.0%, Fig. 1) were respectively purchased from Santa Cruz Biotechnology (USA), Sigma, and Yingxuan Chempharm Co., Ltd (Shanghai, China). Common reagents were purchased from Sinopharm Chemical Reagent (shanghai, China).

Experimental animals

In our methods section, research was granted and approved by Hunan Agricultural University ethics review board. ApoE^{-/-} mice and C57BL/6J mice were respectively bought from Department of Laboratory Animal Science, Beijing University (Beijing, China) and Laboratory Animal Center, College of Veterinary Medicine, Hunan Agricultural University (Changsha, China). In accordance with the Canadian Council on Animal Care (CCAC) guidelines, all animals received humane care. Retained at a constant temperature of 23 ± 1 °C, mice were supplied with high-fat chow (containing 1.25% by weight cholesterol, and 15.8% by weight fat) (Deckert et al. 1999) and water *ad libitum* and exposed to 12 h light / 12 h dark cycle. Body weight and food intake were checked weekly.

Experimental protocols

In vivo, 80 mice at 14 weeks of age were separated casually into four groups (n = 20): C57BL/6J control, ApoE^{-/-}, ApoE^{-/-} + icariin 10 (ApoE^{-/-} treated with 10 mg/kg body wt/day icariin, intragastrically), and ApoE^{-/-} + icariin 30 (ApoE^{-/-} treated with 30

mg/kg body wt/day icariin, intragastrically). Before use, icariin was dissolved in dimethyl sulfoxide (DMSO) as reported previously (Shindel et al. 2010). C57BL/6J mice and untreated ApoE^{-/-} mice were treated with vehicle of icariin (30 mg/kg per day). After 6 weeks, mice were anesthetized as described previously (pentobarbital 80 µg/kg IP) (Mundy et al. 2007). Before euthanasia, all mice were fasted nightlong. *In vitro*, primary human umbilical vein endothelial cells (HUVECs) were isolated, grown, and ascertained as reported previously (Hermenegildo et al. 2005). These primary cells were obtained from a pooled population of unique donors or from either single donors. Primary HUVECs were cultured in Dulbecco's modified Eagle's medium containing 100 µg/mL streptomycin, 100 U/ml penicillin, and 10 % (v/v) fetal bovine serums. Primary HUVECs were randomly divided into 7 groups: control, vehicle (10 µmol/L vehicle of icariin), icariin (10 µmol/L icariin), LPC (10 µg/mL LPC), LPC + icariin 1 (LPC plus 1 µmol/L icariin), LPC + icariin 3 (LPC plus 3 µmol/L icariin), and LPC + icariin 10 (LPC plus 10 µmol/L icariin). Cell damage was elicited by LPC (10µg/ml) for 24 h. Icariin was dissolved in DMSO. Before exposed to LPC for 24 h in the presence of icariin, HUVECs were exposed to icariin (1, 3 or 10 µM) for 1 h. At the end of study, samples of aorta and plasma were obtained from the mice. NO and reactive oxygen species (ROS) production in the conditioned medium and plasma, eNOS expression in aorta and HUVECs were analyzed.

Determination of plasma lipid concentration

According to the manufacturer's recommendations, plasma total cholesterol (TC) concentration, plasma triglyceride (TG) concentration was determined by enzymatic method (bioMerieux, Lyon, France) using an automated analyzer (Type 7170A, Hitachi)

Measurement of ROS

According to the detail described previously (Barry-Lane et al. 2001; Steffen et al. 2012), changes in aortic and intracellular ROS levels were determined by dihydroethidine (DHE). Mean data *in vivo* for the quantification of fluorescence were showed as intensity per μm^2 in aortic sections. Results *in vitro* were expressed as the mean fluorescence intensity (arbitrary units).

Analysis of mRNA expressions of eNOS

Real-time PCR was performed to quantify eNOS mRNA. Using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was isolated from aorta and HUVECs, thus reverse-transcribed. The following primer pairs were used: mouse eNOS (5'-TTCCGGCTGCCACCTGATCCTAA-3' forward and 5'-AACATGTGTCCTTGC TCGAGGCA-3' reverse) (Limbourg et al. 2002); human eNOS (5'-GTGGCTGTCTG CATGGACCT-3' forward and 5'-CCACGATGGTGACTTTGGCT-3' reverse) (Lai et al. 2003); and mouse GAPDH (5'-GAGAATGGGAAGCTTGTCATC-3' forward and 5'-GTCCACCACCCTGTTGCTGTA-3' reverse) (Limbourg et al. 2002); human GAPDH (5'-CTGCTCCTCCTGTTTCGACAGT-3' forward and 5'-CCGTTGACTCCG

ACCTTCAC-3' reverse) (Monsalve et al. 2007). For comparative purposes, the relative abundance of eNOS / GAPDH in control group was defined as 100%.

Measurement of aortic protein expressions of eNOS

In ice-cold Dulbecco's phosphate-buffered saline, the segregated aorta was homogenized. In SDS sample buffer, cells were lysed. Before the separated proteins transferred to PVDF membranes, same concentrations of protein were separated on a 12% SDS-PAGE. Using a previously described method (Li and Förstermann 2000), the Western Blotting of eNOS expressions were performed.

Analysis of NO concentration

According to the content of nitrite and nitrate, NO level in the plasma and the conditioned medium were obliquely measured as described previously (Feng et al. 2001). In brief, nitrate was transformed into nitrite with aspergillus nitrite reductase, and total nitrite was analyzed using the Griess reagent. Utilizing a spectrophotometer (Shanghai, China), absorbance was analyzed at 540 nm.

Histological evaluation

In buffered formalin (4%), right common carotid arteries were fixed. At room temperature, they were kept for 24 hours. In paraffin, tissue was embedded. Using standard hematoxylin-eosin staining, it was processed for light microscopy (d'Uscio et al. 2001). Morphometric quantification of lesion area was determined on the

computer-digitized images with NIH Image software (Xiao et al. 2011).

Statistical analysis

All values are expressed as means $\pm$ SEM. The significance level was chosen as $P \leq 0.05$. The significance of differences was assessed using ANOVA and Student's *t*-test for unpaired data.

Results

Body weight and plasma lipid concentrations

ApoE^{-/-} mice fed with high-fat chow showed a significant increase in body weight and plasma lipid (TG and TC) concentration, while mice fed with icariin showed a decrease in these data ($P < 0.05$ and $P < 0.01$; Fig. 6).

ROS production

Aortic ROS production was notably enlarged in ApoE^{-/-} mice than in C57BL/6J mice. However, icariin (10 or 30 mg/kg) cure diminished the ROS production in the aorta of ApoE^{-/-} mice ($P < 0.05$ and $P < 0.01$; Fig. 2A). Intracellular ROS production was higher in LPC group than in control group. Pretreatment with icariin (1, 3, or 10 μ M) eliminated the ascent of intracellular ROS production ($P < 0.05$ or $P < 0.01$; Fig. 2B).

eNOS expressions

Aortic eNOS expression was reduced in ApoE^{-/-} mice compared with the C57BL/6J

mouse ($P<0.01$). However, icariin (10 or 30 mg/kg) treatment increased the expression of eNOS in ApoE^{-/-} mice ($P<0.05$ or $P<0.01$) (Fig. 3A). LPC (10 µg/mL) treatment for 24 h induced a large reduction in eNOS expression in HUVECs. Pretreatment with icariin (1, 3, or 10 µM) normalized the degradation of eNOS expression induced by LPC ($P<0.05$ and $P<0.01$; Fig. 3B).

NO concentrations

ApoE^{-/-} mice showed a large decrease in NO plasma concentration compared with control mice. There were more plasma NO concentrations in icariin group than in model group ($P<0.05$ or $P<0.01$; Fig. 4A). LPC (10 µg/ml) treatment caused a big decline in NO level in the conditioned medium. This change was reversed by icariin (1, 3, or 10 µM) pretreatment ($P<0.05$ and $P<0.01$; Fig. 4B).

Histological examination

Histological examination revealed that no obviously atherosclerotic changes were found in the carotid arteries of C57BL/6J mice. ApoE^{-/-} mice had significantly more atherosclerosis in the aortic root. Supplementation of icariin to ApoE^{-/-} mice attenuated the development of atherosclerosis ($P<0.05$ and $P<0.01$; Fig. 5).

Discussion

NOS generates L-arginine to NO. There are three different NOS forms including eNOS, neuronal NOS, and inducible NOS (iNOS). Neuronal NOS, eNOS, and iNOS

are respectively found in neurons, endothelial cells, and macrophages. Although excessive NO production by iNOS induces or aggravates disease, eNOS-produced NO at a physiologic level is favorable (Zanetti et al. 2000). Furthermore, reduced endothelial NO level is often related to reduced activity and expression of eNOS, which may contribute to the atherosclerosis. These findings show that the eNOS / NO pathway may be a valuable pharmacologic aim for regulating atherosclerosis.

Many experiments have shown eNOS / NO pathway is inhibited by oxidant stress. Pharmacological approaches as well as molecular mechanisms involved in oxidative stress under pathological conditions are reported. Together with a local elevated degradation of NO by enhanced generation of ROS with subsequent cascade of oxidation-sensitive mechanisms in the arterial wall, endothelium injure stimulated by atherosclerosis results in the reduction of eNOS bioactivity with subsequent impaired release of NO (Napoli et al. 2006). In addition, treatment of endothelial cells with LPC significantly reduced the level of NO, expression of eNOS (Zhao et al. 2007). The present results also suggested that LPC significantly increased ROS generation accompanied with a big decline in NO level in the conditioned medium and eNOS expression *in vitro*. Aortic ROS production was notably enlarged in ApoE^{-/-} mice concomitantly with a decrease in eNOS expression and NO level. What is more, oxidation of tetrahydrobiopterin induces eNOS uncoupling and thus potentiation of oxidative stress and decline in eNOS-derived NO (Li et al. 2014). Recent study reports that oxidative stress causes temporal perturbations in biopterin ratio that changes eNOS from coupled state to an uncoupled state (Joshi et al. 2015). Our

study showed that the produce of ROS was abrogated by icariin *in vivo* and *in vitro*. From what has been mention above, we may come to the conclusion that the possible mechanisms by which icariin improved the eNOS/NO pathway is related to prohibition of oxidative stress in the present study.

Icariin has cardiovascular pharmacological effects. Icariin can attenuate the atherosclerosis by its lipid-lowering effects (Zhang et al. 2013), inhibiting foam cell formation (Yang et al. 2015), and protecting erythrocytes against free-radical-induced peroxidation (Liu et al. 2004). However, the connection between beneficial effect of icariin on atherosclerosis and eNOS / NO pathway had not been studied previously. Icariin has antioxidant effect. Zhao et al have reported that icariin defends against oxidative DNA damage stimulated by AAPH (Yang et al. 2015). Wang et al have showed that icariin improves H₂O₂-stimulated oxidative damages of ECV-304 cells (Wang and Huang 2005). In the present study, icariin treatment efficiently led to an increase in NO level, a reduction in lesion area, ROS productions, and an elevation in the expression of eNOS *in vivo*. Icariin treatment also significantly resulted in a reduction in ROS generation, and an elevation in eNOS expression and NO level *in vitro*. These findings suggested that icariin could improve atherogenesis, and the favorable effect was related to its ability to modulate the eNOS / NO pathway by restraining oxidative stress.

It is noteworthy that icariin has lipid-lowering effects and may treat and prevent the thrombosis in the atherosclerotic process in rabbits fed a high-cholesterol diet (Zhang et al. 2013). In the present experiment, the large reduction in TC and TG

levels together with increased aortic eNOS expression, and plasma NO concentration also induced by icariin in ApoE^{-/-} mice. It has been reported that NO takes part in modulation of lipid metabolism. Lower NO synthesis leads to enrichment in hepatic TG production by lower fatty acid oxidation and higher esterification of fatty acid, resulting in the elevation of very low density lipoprotein-TG (Goto et al. 1999). Furthermore, chronic administration with L-N-nitroarginine (a NO synthase inhibitor) caused hyperlipidemia in rats (Khedara et al. 1996). Exogenous ADMA (an endogenous NOS inhibitor) treatment enhanced plasma TG level in ApoE^{-/-} mice (Xiao et al. 2007). Therefore, it has been suggested that icariin decrease plasma TG and TC level by elevating NO production.

In addition, body weight lowered in icariin treated mice in the current study. It has been reported that icariin inhibit lipid deposition during adipocyte differentiation of 3T3-L1 preadipocytes (Han et al. 2016). It is probable that icariin decreases body weight, which is associated with reduced-fat accumulation. Much further work is needed to explain the mechanisms involved in this regulation.

In conclusion, our study shows that icariin improves eNOS / NO pathway to prohibit the atherogenesis of ApoE^{-/-} mice.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Figure Legends

Fig. 1. Chemical structure of icariin.

Fig. 2. Body weight and plasma lipid concentration. (A) Body weight; (B) Plasma total cholesterol concentration; (C) Plasma triglyceride concentration. Data are means $\pm$ S.D., $n = 10$. **, Significant at $P < 0.01$, compared with control. +, $P < 0.05$ and ++, $P < 0.01$ compared with ApoE^{-/-}. ApoE^{-/-}, apolipoprotein E-deficient; Icariin, Icariin at (L) 10 mg/kg or (H) 30 mg/kg.

Fig. 3. ROS production. (A) ROS production in mice aorta. (B) Intracellular ROS concentration. Data are means $\pm$ S.D., Data are means $\pm$ S.D., $n = 10$ in *vivo*, $n = 3-4$ in *vitro*. **, Significant at $P < 0.01$, compared with control. +, $P < 0.05$ and ++, $P < 0.01$ compared with ApoE^{-/-} or LPC. ApoE^{-/-}, apolipoprotein E-deficient; ROS, reactive oxygen species; LPC, lysophosphatidylcholine; Icariin, Icariin at (L) 10 mg/kg or (H) 30 mg/kg in *vivo*; Icariin at (L) 1 μ mol/L or (M) 3 μ mol/L or (H) 10 μ mol/ in *vitro*.

Fig. 4. eNOS expression. (A) aortic eNOS expression; (B) eNOS expression in vitro. Data are means $\pm$ S.D., Data are means $\pm$ S.D., $n = 10$ in *vivo*, $n = 3-4$ in *vitro*. **, Significant at $P < 0.01$, compared with control. +, $P < 0.05$ and ++, $P < 0.01$ compared with ApoE^{-/-} or LPC. ApoE^{-/-}, apolipoprotein E-deficient; eNOS, endothelial nitric oxide synthase; LPC, lysophosphatidylcholine; Icariin, Icariin at (L) 10 mg/kg or (H) 30 mg/kg in *vivo*; Icariin at (L) 1 μ mol/L or (M) 3 μ mol/L or (H) 10 μ mol/ in *vitro*.

Fig. 5. NO concentrations. (A) Plasma NO concentration; (B) NO concentrations in conditioned medium. Data are means $\pm$ S.D., Data are means $\pm$ S.D., $n = 10$ in *vivo*, $n = 3-4$ in *vitro*. **, Significant at $P < 0.01$, compared with control. +, $P < 0.05$ and ++, $P < 0.01$ compared with ApoE^{-/-} or LPC. ApoE^{-/-}, apolipoprotein E-deficient; MDA, malondialdehyde; NO, nitric oxide; LPC, lysophosphatidylcholine; Icariin, Icariin at (L) 10 mg/kg or (H) 30 mg/kg in *vivo*; Icariin at (L) 1 μ mol/L or (M) 3 μ mol/L or (H) 10 μ mol/ in *vitro*.

Fig. 6. Histological Evaluation. (A) Hematoxylin-eosin staining of aortic atherosclerotic lesions ($\times 100$) (B) Atherosclerotic lesions area. Data are means $\pm$ S.D., $n = 5$. **, Significant at $P < 0.01$, compared with control. +, $P < 0.05$ and ++, $P < 0.01$ compared with ApoE^{-/-}. ApoE^{-/-}, apolipoprotein E-deficient; Icariin, Icariin at (L) 10 mg/kg or (H) 30 mg/kg.

Figure 1

Figure 2

Figure 3

(A)

(B)

Figure 4

(A)

Figure 4

(B)

Figure 5

Figure 6

(A)

(B)