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Ursolic Acid induces Allograft Inflammatory Factor-1 Expression via Nitric Oxide-related Mechanism and Increases Neovascularization

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ABSTRACT

Ursolic acid (UA), a triterpenoid compound found in plants that used in the human diet and in medicinal herbs, possesses a wide range of biological benefits including anti-oxidative, anti-inflammation, and anti-carcinogenesis effects. Endothelial expression of allograft inflammatory factor-1 (AIF-1) mediates vasculogenesis and nitric oxide (NO) produced by endothelial NO (eNOS) represents a vascular protection principle. It is unclear whether UA affects the neovascularization mediating by AIF-1 and eNOS expression. This study investigated the effects and mechanisms of UA on angiogenesis *in vivo* in hind limb ischemic animal models and *in vitro* in human coronary artery endothelial cells (HCAECs). In this study, we tried to explore the impact of UA on ECs activities *in vitro* in human coronary endothelial cells, vascular neovasculogenesis *in vivo* in mouse hind-limb ischemia model, and the possible role of AIF-1 in the vasculogenesis. The results demonstrated that UA enhances collateral blood flow recovery through induction of neovascularization in a hind limb ischemia mouse model. The *in vitro* data show that UA increases tube formation and migration capacities in human endothelial cells; exposing HCAECs to UA increased AIF-1 expression through NO-related mechanism. Moreover, UA administration increased capillary density, eNOS and AIF-1 expression in ischemic muscle. These findings suggest that UA may act as a therapeutic agent in the induction of neovascularization and provide a novel mechanistic insight into the potential effects of UA on the ischemic vascular diseases.

KEYWORDS: Allograft inflammatory factor-1; Chinese herbal medicine; Endothelial nitric oxide synthase; Neovascularization; Ursolic acid

INTRODUCTION

Ursolic acid (3β-hydroxy-urs-12-en-28-oic-acid) (UA) is a triterpenoid compound found in plants that used in the human diet and in medicinal herbs (*1, 2*), in the form of the free acid or as aglycones of triterpenoid saponins (*3*). It is well known that UA possesses a wide range of biological benefits including anti-oxidative stress (*4, 5*), anti-inflammation (*5*), and anti-carcinogenesis effects (*6*). Increasing evidences suggest that UA may against free-radical-induced lipid peroxidation (*7*), increase the activities of endothelial nitric oxide synthase (eNOS) as well as inhibit nicotinamide adenine dinucleotide phosphate oxidase 4 expression (*8*), enhance the nonenzymatic antioxidative activities (*4*), and regulate high glucose-induced apoptosis (*9*). Resulting from the pleiotropic benefits, administration of UA significantly reduced the isoproterenol-induced myocardial ischemia, hyperglycemia-induced monocytic apoptosis (*9*), ethanol-mediated liver and heart damage (*10, 11*), and D-galactose-induced neurotoxocity in brain (*12*). Additionally, the anti-inflammation of UA had been explored in previous. These results showed that UA may inhibit the metabolism of arachidonate (*13*), attenuate inducible NOS and cyclooxygenase-2 expression (*14*) as well as prostaglandin E2 synthesis (*15*). Therefore, administration of UA may efficaciously prevent the symptoms as redness, edema, heat and pain. These findings suggest that the pharmacological action of UA may offer therapeutic strategies for treatment of inflammatory and oxidative stress-related disorders.

Notwithstanding the anti-inflammation and anti-oxidative stress capability, the effects of UA on angiogenesis ability of endothelial cells (ECs) were controversial in previous studies. Casimiro *et al.* had demonstrated *in vitro* that UA can inhibit endothelial cell proliferation, migration, and differentiation which inhibiting angiogenesis, but simultaneously UA may increase the expression of urokinase and

matrix metalloproteinase-2 (MMP-2) which triggering angiogenesis (*16*). Further, Kiran *et al*. presented that treatment with UA increased the expression of E-selectin, intracellular adhesion molecule (ICAM), vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF-2) in human umbilical vein endothelial cells (HUVECs) (*17*). Thus, intensively to study the effects of UA on angiogensis *in vitro* appropriate to human cells and *in vivo* in animal models is necessary.

In 1995, Utans *et al.* had identified and characterized a novel macrophage factor, allograft inflammatory factor-1 (AIF-1), at the first time in the cytokine-rich milieu of cardiac allografts; upregulation of AIF-1 may accelerate the infiltration in INF-γ-stimulated macrophages which is associated with chronic rejection (*18*). Further, Increasing evidences demonstration that inhibition of AIF-1 may significantly reduce the macrophages and vascular smooth muscle cells activation and signal transduction during atherogenesis (*19-22*). AIF-1 plays key roles in vascular smooth muscle cells and macrophages, which involving in vascular inflammation. In contrast, the expression and roles of AIF-1 in vascular ECs were uncharacterized until 2009. In 2009, Tian *et al.* firstly demonstrated that AIF-1 expression in ECs which mediating vasculogenesis (*23*). Further more, Jia *et al.* revealed that overexpression of AIF-1 promotes cell cycle transition probably by upregulation of basic fibroblast growth factor (*24, 25*). Therefore, in this study, we tried to explore the impact of UA on ECs activities *in vitro*, vascular neovasculogenesis *in vivo*, and the possible role of AIF-1 in the vasculogenesis.

MATERIALS and METHODS

Reagents

UA (≥ 98.5% purity) and other reagents were purchased from Sigma-Aldrich Co. (St. Louis, OM, USA). UA was dissolved in ethanol as a 10 mM stock solution, and stored at 4°C.

Animals

All animals were treated according to protocols approved by the Institutional Animal Care Committee of the Taipei Medical University (Taipei, Taiwan). The experimental procedures and animal care conformed to the ‘‘Guide for the Care and Use of Laboratory Animals’’ published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male outbred ICR mice at 6-8 weeks old were purchased from the National Laboratory Animal Center in Taiwan. All mice were kept in microisolator cages on a 12-h day/night cycle and on a commercial mouse chow diet (Scientific Diet Services, Essex, UK) as well as with water *ad libitum*.

Thirty mice were used. The animals were divided into six groups. Group 1 was the naïve control; group 2 received hind limb ischemia operation at the end of week 1 whine experiment; group 3 received hind limb ischemia operation at the end of week 1 and intra-peritoneal injections of UA (2 mg/kg body weight) at the end of week 1, 2, and 3; group 4 received hind limb ischemia operation at the end of week 1 and intra-peritoneal injections of UA (5 mg/kg body weight) at the end of week 1, 2, and 3; group 5 received intra-peritoneal injections of UA (5 mg/kg body weight) only at the end of week 1, 2, 3; group 6 received incision but did not receive hind limb ischemia operation (sham control). We established the dose of UA based on our pilot data, which was performed according to previous animal study (Atherosclerosis. 2006

Jan;184(1):53-62). The acute toxicity (LD50) of UA on rodent-mouse are > 637 mg/kg (intra-peritoneal) and 8330 mg/kg (oral), respectively. The pharmacokinetic parameters of UA in Rats after oral administration (80.32 mg/kg) are C_{max} (294.8 ng/ml), t_{max} (1.0 h), K_e (0.16/h), $t_{1/2}$ (4.3 h), respectively (Yakugaku Zasshi. 2005 Jun;125(6):509-15). The pharmacokinetic data from Liao *et al*. suggest the absorption of UA was rapid, but its concentrations in rat plasma were extremely low after oral administration. This implies that UA has high binding activity in organs and low blood distribution; another possibility was the low bioavailability of UA because it is metabolized by the gut wall and liver on one hand, and on the other hand, it is poorly absorbed by the intestine (Yakugaku Zasshi. 2005 Jun;125(6):509-15).

Mouse Hind Limb Ischemic Experiment

Six-week-old male ICR mice on a commercial mouse chow diet. After 1 week whine experimental, unilateral hind limb ischemia was induced by excising the right femoral artery as previously described (*26*). Briefly, the animals were anesthetized by intraperitoneal injection of Xylocaine (2 mg/kg body weight) plus Zoletil (containing a dissociative anaesthetic, Tiletamine and Zolazepam with ratio 1:1; 5 mg/kg body weight). The proximal and distal portions of the femoral artery were ligated. Hind limb blood perfusion was measured with a Laser Doppler perfusion imager system (Moor Instruments Limited, Devon, UK) before and after the surgery and then followed weekly. The animals were sacrificed at the end of the forth experimental week. To avoid the influence of ambient light and temperature, the results were expressed as the ratio of perfusion in the right (ischemic) versus left (non-ischemic) limb.

Biochemical Measurements

Blood samples for biochemical measurements were collected from each animal before, at 1, 2, 3, and 4 weeks after the start of experiment. Samples were collected from the mandibular artery into sodium citrate containing tubes and separated by centrifugation. Serum blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were also measured using SPOTCHEMTM automatic dry chemistry system (SP-4410, Arkray, Japan).

Morphmetry and Immunohistochemistry

The liver, kidney, and whole ischemia limbs were then harvested, the adhering tissues and femora were carefully removed, immersion-fixed with 4% buffered paraformaldehyde, performed on serial 5-μm-thick paraffin-embedded sections. Hematoxylin/eosin staining was used for liver and kidney morphmetry. Immunohistochemical staining was performed from mouse ischemia skeleton muscle using a goat anti-CD31 (Santa Cruz, CA, USA), rabbit anti-eNOS (Millipore, MA, USA), rabbit anti-phospho eNOS (Millipore, MA, USA), and goat anti-AIF-1 (Abcam, CA, USA) antibodies.

Cell Culture

Human coronary artery endothelial cells (HCAECs) were purchased from Cascade Biologics and in USA and were grown in endothelial cell growth medium (medium 200, Cascade Biologics) supplemented with 2% fetal bovine serum (FBS), 1 μg/ml of hydrocortisone, 10 ng/ml of human epidermal growth factor, 3 ng/ml of human fibroblast growth factor, 10 μg/ml of heparin, 100 U/ml of penicillin, 100 pg/ml of streptomycin, and 1.25 mg/ml of Fungizone (Gibco, NY, USA). HCAECs were

grown at 37℃ in a humidified 5% CO_2 atmosphere and were used at passages 3-8. The growth medium was changed every other day until confluence. The purity of HCAECs cultures was verified by immunostaining with a monoclonal antibody directed against endothelial-specific human von Willebrand factor (vWF; R&D Systems, MN, USA).

Measurement of Cytotoxicity and Proliferation by MTT Assay

Cell cytotoxicity of UA was analyzed by the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay. HCAECs ($2x10^4$ cells) were grown in 96-well plates and incubated with various concentrations (1 - 50 μM) of UA for 24 h. Subsequently, MTT (0.5 μg/ml) was applied to cells for 4 h to allow the conversion of MTT into formazan crystals. After washing with phosphate-buffered saline, the cells were lysed with dimethyl sulfoxide (DMSO), and the absorbance read at 530 nm using a DIAS Microplate Reader (Dynex Technologies, VA, USA).

Endothelial Cell Tube Formation Assay

Tube formation assay was performed on HCAECs to assess the capacity of vasculogenesis, which is believed to be important in new vessel formation. *In vitro* tube formation assay was performed with Angiogenesis Assay Kit (Chemicon, CA, USA)(*27*). The protocol was according to the manufacturer's instructions. In brief, ECMatrix gel solution was thawed at 4°C overnight, then mixed with ECMatrix diluent buffer, and placed in a 96-well plate at 37°C for 1 h to allow the matrix solution to solidify. HCAECs were harvested as described above with trypsin/EDTA, then HCAECs (10^4 cells) were placed on matrix solution with M200 medium with UA, and incubated at 37°C for 12 h. Tubule formation was inspected under an

inverted light microscope. Four representative fields were taken and the average of the total area of complete tubes formed by cells was compared by computer software, Image-Pro Plus.

Cell Wound-healing Assay

The migration ability of UA-treated HCAECs cells were assayed in a monolayer denudation assay as described (*28*). HCAECs were cultured in a 12-well plate. The confluent cells ($2x10^5$ cells/well) were wounded by scraping with a 100-μL pipette tip, which denuded a strip of the monolayer that is 300 μm in diameter. The cultures were washed twice with PBS, then the medium supplemented with 5% FBS was added and the rate of wound closure was observed after 24 hours. The distance of the gap was measured under a 4x phase objective of a light microscope (OLYMPUS IX71, USA), monitoring with a CCD camera (Macro FIRE 2.3A), and captured with a video graphic system (Picture Frame Application 2.3 software).

F-actin Staining and Immunofluorescent Staining

HCAECs seeded on the cover slide ($1x10^5$ cells) and fixed with 4% paraformaldehyde as well as permeabilized for 10 min with 0.1% Triton X-100 solution. Cells were blocked the non-specific antigen with 2% BSA for 30 min, and stained with rhodamine conjugated phallodin or goat anti-AIF-1 (Abcam, CA, USA) antibody and then incubated with the secondary antibody conjugated to fluorescein isothiocyanate (FITC). The 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) was using for identification of nucleus. The slides were observed with confocal microscopy.

Western Blot Analysis

Cells were lysed with lysis buffer (0.5 M NaCl, 50 mM Tris, 1 mM EDTA, 0.05 % SDS, 0.5 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min at 4ºC, then the cell lysates were centrifuged at 4,000 *g* for 30 min at 4ºC. Protein concentrations in the supernatants were measured using a Bio-Rad protein determination kit (Bio-Rad, Hercules, CA). The supernatants were subjected to 10% SDS-PAGE, then transferred for 1 h at room temperature to polyvinylidene difluoride (PVDF) membranes, which were then treated for 1 h at room temperature with PBS containing 0.05% Tween 20 and 2% skimmed milk and incubated separately for 1 h at room temperature with mouse anti-eNOS (Millipore, MA, USA), or goat anti-human AIF-1 antibodies (Abcam, CA, USA). After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-goat or mouse IgG. Immunodetection was performed using chemiluminescence reagent and exposure to Biomax MR Film (Kodak, USA).

Statistical Analyses

Values were expressed as means ± SEM. Statistical evaluation was performed using Student's *t*-test and one- or two-way ANOVA followed by Dunnett's test. A probability value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

UA Enhances Recovery of Capillary Density in ICR Mice

During the experimental period, weight gain and final weight did not differ significantly between the groups of animals (data not shown). As shown in **Table 1**, serum BUN, creatinine, and ALT levels also showed no significant difference between groups even though the AST level were increased in receiving hind limb ischemic groups at the ends of week 2. In order to measure the dosage of UA using in present animal study is harmless, the morphmetry analysis of kidney and liver were performed. Comparing with naïve control group, after intraperitoneal administration of 5 mg/kg BW UA for 3 times whine 4 weeks did not cause kidney injury, including glomerulonephritis, compression of capillaries, and narrowing of Bowman space in experimental mice (**Figure 1A**). The liver samples of UA administrated mouse did not show the feathery degeneration, micro and macro cellular fatty changes, periportal fibrosis and vascular congestion (**Figure 1B**). These results indicated that mice treated with 5 mg/kg BW of UA demonstrated normal kidney and liver function as well as histology.

To evaluate the angiogenic effect of UA, we induced tissue ischemia by unilateral hind limb ischemia surgery in wild-type male ICR mice (n = 5 for each group). As shown in **Figure 2A and 2B**, the hind limb ischemic mice showed delayed blood flow recovery after ischemia surgery compared with that in control mice as determined by laser Doppler imaging, while intraperitoneal injection of 2 and 5 mg/kg BW UA significantly improved blood flow in hind limb ischemia-treated mice post ischemic surgery for 2 weeks (the end of week 3). The blood flow of the limb did not significantly change in control, only UA-treated, and only incision-received mice during the period of experiment (**Figure 2B**). After ischemic surgery for 3 weeks (the

end of week 4 of the experiment), the ischemia/normal perfusion ratio in UA-treated group were higher than that in non-UA-treated group (**Figure 2C**).

Consistent with the measurements by Laser Doppler imaging, anti-CD31 immunostaining revealed that administration of UA significantly increased the number of detectable capillaries in the ischemic muscle, both in 2 mg/kg BW UA- and 5 mg/kg BW UA-treated ischemic mice (**Figure 2D**) than that in non-UA-treated ischemic mice. These results indicating that UA treatment may enhance recovery of capillary density after hind limb ischemia in ICR mice.

UA Increases Proliferation, Tube Formation and Migration of HCAECs

In order to analyze the cell viability and cytotoxicity of UA, the MTT assay was performed. The **Figure 3** showed that UA treatment of HCAECs with 1 μM for 12 and 24 h, or 2.5 μM for 12 h did not result in cell viability change or cell cytotoxicity. In contrast, more than 5 μM UA may cause a significant reduction in cell viability. Interestingly, treated HCAECs with UA in 2.5 μM for 24 h, and 5 μM for 12 h or 24 h may significant induce the increasing of cell proliferation (125.4 ± 12.5%, 120.6 ± 14.3%, 132.8 ± 6.5% of control, respectively). The results indicated that moderate concentration (2.5 - 5 μM) of UA may increase the HCAECs proliferation even though the higher concentration induced the cell cytotoxicity.

To explore the potential effects of UA on ECs neovascularization, the tube formation and migration assays were performed on HCAECs. It has been shown that ECs make capillary network formation on Matrigel successfully (*16*). After 24 h of 2.5 or 5 μM UA culturing, the functional capacity for tube formation of HCAECs on ECMatrix gel were significantly increased compared with the control group (200.1 ± 36.9%, 258.4 ± 28.4% of control, respectively)(**Figure 4A and 4B**). Previous study

demonstrated that eNOS regulates angiogenesis associated with oxidative stress in response to tissue ischemia (*29*). Incubation with 10 μM of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) for 24 h significantly increased tube formation function in HCAECs, whereas preincubation with NOS inhibitor $_{L}$-N^{g}-nitro-$_{L}$-arginine methyl ester (L-NAME) for 2 h decreased the effect of UA on tube formation. Treatment of cells with UA at 2.5 or 5 μM as well as SNAP at 10 μM for 24 h induced their wound-healing/migratory abilities in a dose-dependent manner (200.1 ± 36.9%, 258.4 ± 28.4%, 290.9 ± 24.1% of control, respectively). After treatment with L-NAME (10 μM) for 2 h, the migratory abilities of HCAECs were significantly reduced (109.3 ± 12.4% of control), as compared with that of the UA-treated group (**Figure 4C and 4D**).

Cytoskeleton is a key component for cells in order to maintain their proliferation and migration(*30, 31*). Therefore, we studied the cytoskeleton organization of HCAECs treated with the UA for 24 h. Cells were then fixed and stained for F-actin using phallodin-rhodamine. **Figure 4E** showed that naïve HCAECs presented a cobblestones pattern and the distribution of F-actin were crisscrossed. However, both treatment of 2.5-5 μM UA and 10 μM SNAP showed the presence of stress fibers that were visible parallel (white arrow) and crossing the cell from one side to the other. Additionally, the HCAECs displayed peripheral bands and cluster of F-actin (arrow head) in the margin region as well as individual stress fibers disappeared partly (double arrow) indicating the redistribution of F-actin. Pretreatment of L-NAME may reverse the effect of UA on F-actin reorganization. These results suggested that UA might be an effective stimulator for the tube formation and migration of HCAECs which mediating by NOS activity and cytoskeleton reorganization.

UA is the major component of some traditional medicine herbs and is well

known to possess a wide range of biological functions, such as anti-oxidative, anti-inflammation, and anti-cancer activities. In contrast to these beneficial properties, some laboratory studies have recently revealed that the effects of UA on normal cells and tissues are occasionally pro-inflammatory (*32*). On the other hand, little information is available for the angiogenic effect of UA. Sohn *et al.* firstly examined the anti-angiogenic activity of UA by using the chick embryo chorioallantoic membrane assay. They also tested for inhibitory effect on the proliferation of bovine aortic endothelial cell. Based on the results, they speculated that the inhibitory effects on bovine aortic endothelial cell proliferation of UA might be important for anti-angiogenesis (*33*). Cárdenas *et al.* then study the effects of UA on different key steps of angiogenesis; UA is able to inhibit key steps of angiogenesis *in vitro*, including endothelial cell proliferation, migration, and differentiation. At the same time, it seems to stimulate other key steps of angiogenesis, such as extracellular matrix degradation by MMP-2 and urokinase (*17*). More recently, UA was found to inhibit the tumor-associated capillary formation in mice induced by highly metastatic melanoma cells. Nontoxic concentrations of UA reduced vessel growth from the rat aortic ring, inhibited proliferation, migration, and invasion of ECs. Gelatin zymographic analysis showed the inhibitory effect of UA on the protein expression of matrix metalloproteinases MMP-2 and MMP-9 (*34*). The above observation shows the anti-angiogenic activity of UA. By contract, Kiran *et al.* reported that upregulation of angiogenic modulators in ECs treated with UA; low concentration of UA (< 10 μM) had no effect on these modulators. The cells were maintained in culture in serum free un-stimulated conditions. The changes in the angiogenic modulators correlated with the ability of UA to influence angiogenesis. Treatment with UA at higher concentrations promoted angiogenesis which was evidenced by tube formation of c

HUVECs and greater extent of endothelial sprouting in rat aortic rings; but no such effects were observed at low concentration. However treatment of HUVECs and aortic rings, stimulated by maintaining in medium supplemented with serum, with UA caused inhibition of angiogenic phenotype (*17*). Our present study showed that UA enhances collateral blood flow recovery through induction of neovascularization; low concentration (≤ 5 μM) UA increases tube formation and migration capacities in human ECs. Interestingly, the *in vitro* results indicate that low concentration of UA may increase the HCAECs proliferation while the higher concentration (≥ 10 μM) induced cell cytotoxicity on ECs. Similarly, statins have proangiogenic effects at low therapeutic concentrations but angiostatic effects at high concentrations suggesting that statins have a biphasic dose-dependent effect on angiogenesis (*35-37*). Thus, UA might be also designated as a double-edged sword with both positive and negative effects on angiogenesis, and further evaluations of the effects of UA on the biological status of target cells or tissues are necessary.

UA Increases AIF-1 Expression Through Phosphorylation of eNOS *In Vitro* and Increases Capillary Density, eNOS and AIF-1 Expression *In Vivo*

AIF-1 expression regulates endothelial cell signal transduction mediating proliferation and vasculogenesis (*23, 25*). The **Figure 5A** showed that treatment of 1-5 μM UA for 12-24 h may increase the intracellular AIF-1 expression in HCAECs. Additionally, previous evidences support that vascular eNOS-derived NO is a principle on maintaining vascular physiology (*31, 38*). We therefore investigated the effects of UA on the NO system in HCAECs. After 24 h of incubation, the eNOS phosphorylation at $Ser^{1,177}$ shown by immunoblotting were significantly increased both in HCAECs that had been cultured with 1-5 μM UA-contained medium

compared with that in control conditions (**Figure 5B**). In the other hand, the eNOS phosphorylation in 5 μM UA-treated HCAECs have the time-dependent manner (**Figure 5C**). In order to explore the fact of vascular NO involving in AIF-1 expression, we treated the HCAECs using 10 μM NO donor S-nitroso-N-acetylpenicillamine (SNAP) for 24 h, and the results indicated intracellular AIF-1 expression regulating by NO level. In contrast, coincubation with 10 μM NOS inhibitor $_{L}$-N^{g}-nitro-$_{L}$-arginine methyl ester (L-NAME) significantly decreased the increasing effect of UA on HCAECs (**Figure 5D**). The results consists with Western blot analysis, confocal microscopy revealed that UA and SNAP incubation for 24 h resulted in a marked accumulation of AIF-1; in contrast, treatment with 10 μM of L-NAME may reverse the phenomenon (**Figure 5E**). According to these data indicated that UA may upregulate AIF-1 expression by modulating NO-related mechanisms.

To consistent with the measurements by *in vitro* studies, we performed measurements of eNOS and AIF-1 expression on histological sections harvested from the ischemic hindlimbs of mice. The **Figure 6A** showed that the number of detectable capillaries (white arrow) and eNOS expression (arrow head) from limb ischemia were significantly increased in 2 mg/kg BW and 5 mg/kg BW UA- treated mice compared to controls. According to the images, the eNOS indeed increased in the region of capillaries. We next examined whether UA increased AIF-1 expression in the ischemic hindlimb muscle of mice. As is shown in **Figure 6B**, UA administration may increase the expression of AIF-1 in ischemic muscle compared to controls. These results concluded that treated UA, especially concentrated at 5 mg/kg BW, after hindlimb ischemic surgery may increase the eNOS and AIF-1 expression in the capillaries of muscle.

AIF-1 has been identified in chronic rejection of rat cardiac allografts as well as tissue inflammation in various autoimmune diseases. Kimura *et al.* firstly suggested that AIF-1 is closely associated with the pathogenesis of rheumatoid arthritis which is characterized by massive synovial proliferation, angiogenesis, subintimal infiltration of inflammatory cells (*39*). In addition, EC migration and proliferation have an important role in numerous physiological responses such as angiogenesis (*23*). The expression AIF-1 protein in human arteries with coronary artery vasculopathy and in neointimal cells in animal models of various types of arterial injuries (*22, 40*). AIF-1 expression in ECs of inflamed arteries and can be induced by soluble stimuli in cultured ECs. Inducible AIF-1 expression in ECs suggests an important role for this protein in EC activation; abrogation of AIF-1 expression significantly inhibited EC proliferation and migration (*23*). More recently, Jia *et al.* showed stable introduction of AIF-1 to HUVECs *in vitro* revealed that AIF-1 enhances the proliferation and migration of the EC and promotes G_0/G_1- to S-phase transition (*24*). Taken together, these findings suggest the impact of AIF-1 on EC would stimulate EC activation and angiogenesis and consequently affect the progression of neovascularization in ischemia.

NO produced by eNOS represents a vascular protection principle. NO bioavailability is decreased in atherosclerosis due to increase NO inactivation by reactive oxygen species and reduced NO synthesis. Various types of vascular pathophysiology are associated with oxidative stress that inactivates NO. Also, oxidative stress is likely to be the main cause for oxidation of the essential NOS cofactor, tetrahydrobiopterin (*41*). Aguirre-Crespo *et al.* have demonstrated that UA-mediated vascular relaxation is endothelium dependent, probably due to NO release (*42*). Recently, Steinkamp-Fenske *et al.* identified UA that upregulates eNOS,

and at the same time, reduce NADPH oxidase expression in human ECs through protein kinase C independent mechanisms (*8*). Additionally, UA significantly increased eNOS expression in HUVECs, and enhanced bioactive NO production. Interestingly, UA also been found to reduce the expression of the NADPH oxidase subunit Nox4 and suppressed the production of reactive oxygen species in human endothelial cells (*8*). Our report further showed that UA induces AIF-1 expression via NO-related mechanism and increases neovascularization. To the best of our knowledge, this is the first study show that UA increases AIF-1 expression mediating by phosphorylation of eNOS in HCAECs. On the other hand, since NO is generated by distinct isoforms of NOS [eNOS, neuronal (nNOS), and inducible (iNOS)], the role of iNOS (FEBS Lett. 2001 Dec 7;509(2):156-60) also should be focused vascular protection; the possible role of iNOS in UA-induced vasculogenesis needs to be further investigated.

In conclusion, our results demonstrated that UA enhances recovery of capillary density through induction of neovascularization in a hind limb ischemia mouse model. UA increases tube formation and migration capacities in human ECs; exposing HCAECs to UA increased AIF-1 expression through NO-related mechanism. Moreover, UA administration increased capillary density, eNOS and AIF-1 expression in ischemic muscle.

In the present study, we demonstrated for the first time that triterpenoid compound UA enhances collateral blood flow recovery through induction of neovascularization in a hind limb ischemia mouse model. The *in vitro* data show that UA increases tube formation and migration capacities in human ECs; exposing HCAECs to UA increased AIF-1 expression through NO-related mechanism. Moreover, UA administration increased capillary density, eNOS and AIF-1 expression

in ischemic muscle. These findings suggest that UA may act as a therapeutic agent in the induction of neovascularization and provide a novel mechanistic insight into the potential effects of UA on the ischemic vascular diseases. Further investigation of the regulation underlying UA-induced AIF-1 and NO signaling pathway may contribute to develop a new clinical strategy for ischemic vascular diseases.

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Figure Legends

Figure 1. Control mice treated with UA demonstrated normal kidney and liver histology. Tissue samples were performed on serial 5-μm-thick paraffin-embedded sections. Hematoxylin/eosin staining was used for morphmetry (original magnification ×20). (A) Control mouse kidney showing normal glomerulus (indicated by arrowheads). Following the control group, 5 mg/kg BW UA administration for 3 times whine 4 wks was harmless to kidney. (B) Control mouse liver showing central vein (indicated by black arrow) and hepatocytes arranged in the form of cord. Liver of mouse administrated with UA also showing normal histology.

Figure 2. Effect of UA on blood flow recovery after hind limb ischemia in ICR mice. (A) Representative results of laser Doppler measurements before operation (control), 1 day and 3 weeks after hind limb ischemia surgery in 0, 2 or 5 mg/kg BW UA-administration mice. Color scale illustrates blood flow variations from minimal (dark blue) to maximal (red) values. Arrows indicate ischemic (right) limb after hind limb ischemia surgery. (B) Doppler perfusion ratios (ischemic/non-ischemic hind limb) over time in the different groups. Administration of 2 (■) or 5 (□) mg/kg BW UA provided the beneficial effect of blood flow recovery, which comparing with non-adminstrated group (△) after hind limb ischemia surgery for 2 weeks (the end of week 3 whine experiment). The blood flow of the limb did not significantly change in control (●), only UA-treated (○), and only incision-received mice (▼). Results are means ± SEM. (*$P < 0.05$ compared with non-UA adminstrated group (△). (C) After ischemic surgery for 3 weeks (the end of week 4 of the experiment), the ischemia/normal perfusion ratio in UA-treated group were higher than that in non-UA-treated group. Results are means ± SEM. *$P < 0.05$ was considered

significant. (D) Mice were sacrificed 3 weeks after surgery and capillaries in the ischemic muscles were visualized by anti-CD31 immunostaining.

Figure 3. The effects of UA on cytotoxicity and proliferation were analyzed by the MTT assay. Treatment of HCAECs with 1 - 50 μM of UA for 12 (■) or 24 (□) h, the MTT assay were performed and the absorbance was recorded using a microplate reader. Data are expressed as the mean ± SEM of three experiments performed in triplicate.*$P < 0.05$ compared with control (non-UA treated) at the same time point and considered significant.

Figure 4. The effects of UA on the ability of tube formation and wound-healing in HCAECs. (A) An in vitro angiogenesis assay for HCAECs was used with ECMatrix gel. Representative photos for in vitro angiogenesis are shown. (B) The cells were stained with crystal violet, and the averages of the total area of complete tubes formed by cells were compared by computer software. Data are mean ± SEM; n = 5, *$P <$ 0.05 considered significant. (C) The wound-healing assay for evaluating the effect of UA on HCAECs migration. The HCAECs migrating to the denuded area were counted based on the black base line. The HCAECs were cultured with 2.5 or 5 μM UA, 10 μM SNAP for 24 h, or pretreated with 10 μM L-NAME for 2 h then followed by 5 μM UA before the wound scraping using pipette tip. Photographs were taken after wound scraping for 18 h. (D) The cells that migrated into the denuded area were analyzed; the magnitude of HCAECs migration was evaluated by counting the migrated cells in six random clones under high power microscope fields (×100). Data are expressed as the mean ± SEM of three independent experiments and expressed as the percentage of control. *$P < 0.05$ was considered significant. (E) HCAECs were

treated as in (A). F-actin was stained with rhodamine-phalloidin and the magnitude was evaluated using confocal microscope at 100× fields. The visible parallel stress fibers were indicated as white arrow. The peripheral bands and cluster of F-actin were indicated as arrow head. The disappeared individual stress fibers were indicated as double arrow. The DAPI was used to identify the nuclei of THP-1 cells.

Figure 5. UA increased AIF-1 expression mediating by phosphorylation of eNOS in HCAECs. (A) HCAECs were treated for 12 - 24 h with 1 - 5 μM of UA, the AIF-1 expression were analyzed by Western blot analysis. (B) HCAECs were treated for 18 h with 1 - 5 μM of UA, the eNOS activation (phosphorylation) were analyzed by Western blot analysis. (C) HCAECs were treated for 2-18 h with 5 μM of UA, the eNOS activation were analyzed by Western blot analysis. (D) HCAECs were treated for 24 h with 5 μM of UA, 10 μM of SNAP, or pretreated with 10 μM of L-NAME, for 2 h prior to stimulation with UA for 24 h. the AIF-1 production were analyzed by Western blot analysis. The β-actin and total eNOS protein were used as loading controls. (E) HCAECs were treated as in (D). The intracellular AIF-1 production was analyzed by immunofluorescence and the magnitude were evaluated using confocal microscope at 40× fields. The DAPI was used to identify the nuclei of THP-1 cells.

Figure 6. The eNOS expression combined with capillaries density and AIF-1 expression in ischemic hindlimb tissue evaluated by immunohistochemistry and confocal microscopy at 40x fields. (A) Immunostainning of ischemic hindlimb muscle with anti-CD31 conjugated Alex 633 (red) and anti-eNOS conjugated Alex 488 (green) antibodies. The arrow indicated the endothelium and the arrow head indicated the eNOS in the hindlimb muscle. (B) Immunostainning of ischemic hind limb muscle

with anti-AIF-1 conjugated Alex 488 (green) antibody. The arrow indicated the AIF-1 in the hind limb muscle.