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# Barrier protective effect of asiatic acid in TNF-α-induced activation of human aortic endothelial cells

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ABSTRACT

*Background:* Endothelial cell activation is characterized by increased endothelial permeability and increased expression of cell adhesion molecules (CAMs). This allows monocyte adherence and migration across the endothelium to occur and thereby initiates atherogenesis process. Asiatic acid is a major triterpene isolated from *Centella asiatica* (L.) Urban and has been shown to possess anti-oxidant, anti-hyperlipidemia and anti-inflammatory activities.

*Purpose:* We aimed to investigate protective effects of asiatic acid on tumor necrosis factor-α (TNF-α)-induced endothelial cell activation using human aortic endothelial cells (HAECs).

*Study design:* For cell viability assays, HAECs were treated with asiatic acid for 24 h. For other assays, HAECs were pretreated with various doses of asiatic acid (10–40 μM) for 6 h followed by stimulation with TNF-α (10 ng/ml) for 6 h.

*Methods:* Fluorescein isothiocyanate (FITC)-dextran permeability assay was performed using commercial kits. Total protein expression of CAMs such as E-selectin, ICAM-1, VCAM-1 and PECAM-1 as well as phosphorylation of IκB-α were determined using western blot. The levels of soluble form of CAMs were measured using flow cytometry. Besides, we also examined the effects of asiatic acid on U937 monocyte adhesion and monocyte migration in HAECs using fluorescent-based assays.

*Results:* Asiatic acid significantly suppressed endothelial hyperpermeability, increased VCAM-1 expression and increased levels of soluble CAMs (sE-selectin, sICAM-1, sVCAM-1 and sPECAM-1) triggered by TNF-α. Neither TNF-α nor asiatic acid affects PECAM-1 expression. However, asiatic acid did not inhibit TNF-α-induced increased monocyte adhesion and migration. Interestingly, asiatic acid suppressed increased phosphorylation of IκB-α stimulated by TNF-α.

*Conclusion:* These results suggest that asiatic acid protects against endothelial barrier disruption and this might be associated with the inhibition of NF-κB activation. We have demonstrated a novel protective role of asiatic acid on endothelial function. This reveals the possibility to further explore beneficial effects of asiatic acid on chronic inflammatory diseases that are initiated by endothelial cell activation.



## Introduction

Atherosclerosis is a chronic inflammatory disease initiated by endothelial dysfunction, which ultimately develops into coronary heart disease. Hyperlipidemia, diabetes and hypertension are risk factors known to initiate atherogenesis. In early stage of atherosclerosis, endothelial dysfunction is accompanied by activation of endothelial cells that involves a complex interplay between leukocytes, endothelial cells and cytokines (Sitia et al. 2010). In response to pro-inflammatory stimuli, the surface expression of cell adhesion molecules (CAMs) such as E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 are up-regulated in endothelial cells. These favor the recruitment of circulating leukocytes and hence promote firm attachment between leukocytes and endothelial cells. In addition, ICAM-1 and VCAM-1 serve as signaling molecules that promote the production of reactive oxygen species (ROS), a key player of

*Abbreviations:* HAECs, human aortic endothelial cells; TNF-α, tumor necrosis factor-α; CAMs, cell adhesion molecules; sCAMs, soluble form of cell adhesion molecules; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule-1; sE-selectin, soluble E-selectin; sICAM-1, soluble ICAM-1; sVCAM-1, soluble VCAM-1; sPECAM-1, soluble PECAM-1; NF-κB, nuclear factor-κB; IκBα, inhibitor of NF-κB alpha; FITC-dextran, fluorescein isothiocyanate conjugated-dextran.

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TNF-$\alpha$-induced increased permeability (van Wetering et al. 2003; Wolf et al. 2013). Following the binding of monocytes to endothelium, platelet endothelial cell adhesion molecule (PECAM)-1 regulates the transmigration of monocytes across the blood vessel wall. Enzymatic cleavage of the surface CAMs results in the secretion of soluble form of cell adhesion molecules (sCAMs) such as soluble E-selectin (sE-selectin), soluble ICAM-1 (sICAM-1), soluble VCAM-1 (sVCAM-1) and soluble PECAM-1 (sPECAM-1) (Leeuwenberg et al. 1992). The level of sICAM-1 is elevated in hyperlipidemic individuals and associated with high cardiovascular risk (Karasek et al. 2005). Importantly, an increase in endothelial permeability to small molecules occurs in parallel with the diapedesis of monocytes, leading to disruption of endothelial barrier that subsequently ease the foam cell formation (Funk et al. 2012). Therefore, natural compounds that could protect against early atherogenic events, which occur before the formation of atherosclerotic lesions, might have beneficial effects in preventing atherosclerosis.

Tumor necrosis factor-alpha (TNF-$\alpha$), a pro-inflammatory cytokine, is found to be expressed in atherosclerotic lesions (Barath et al. 1990). During the progression of atherosclerosis, TNF-$\alpha$ sustains and propagates the inflammatory response by increasing the surface expression of CAMs, stimulating the production of inflammatory cytokines and chemokines as well as enhancing endothelial permeability. Although the molecular pathways that lead to TNF-$\alpha$-induced increased permeability are not well understood, accumulating evidence in recent years has suggested that the CAMs are central mechanisms in mediating endothelial hyperpermeability stimulated by TNF-$\alpha$ (Frank and Lisanti 2008; Marcos-Ramiro et al. 2014).

Nuclear factor-$\kappa$B (NF-$\kappa$B) family consists of a group of transcription factors that regulate various cellular processes such as inflammation, immune response and programmed cell death. In cytoplasm, these transcription factors bind with an inhibitory protein, inhibitor of NF-$\kappa$B-alpha (I$\kappa$B$\alpha$), preventing them from entering the nucleus. Upon activation, I$\kappa$B$\alpha$ phosphorylates and undergoes degradation while releasing the bound NF-$\kappa$B dimers into the nucleus. The transcription cascades of many pro-inflammatory genes are then being initiated. Thus, inhibition of the NF-$\kappa$B pathway might be a promising therapeutic strategy to prevent inflammatory diseases that are perpetuated by cytokines.

Asiatic acid is one of the pentacyclic triterpenoids isolated from *Centella asiatica* (L.) Urban., a traditional medicinal plant that is commonly found in swampy areas of most tropical countries including India, China, Indonesia, Malaysia and other Asian countries. *C. asiatica* is well known for its wound healing and neuroprotective effects in Ayurvedic medicine. In Malaysia and Thailand, it is consumed as raw vegetables or blended into juice and served as tonic drinks (Hashim 2011). The lipid lowering effects of *C. asiatica* extract were previously reported by other groups. *C. asiatica* extract has been shown to reduce total cholesterol, triglyceride and plasma glucose levels in hyperlipidemic rats (Supkamonseni et al. 2014). A fraction of ethanol extract of *C. asiatica* was reported to improve lipid profiles in chemical-induced hyperlipidemic mice and high fat diet induced-hamster models (Zhao et al. 2014). In several clinical studies, total triterpenic fraction of *C. asiatica* (TTFCA) has been demonstrated to prevent the progression of atherosclerotic plaques in asymptomatic patients when administered in combination with Pycnogenol, a pine bark extract (Belcaro et al. 2015a; Belcaro et al. 2015b). In particular, TTFCA has been shown to improve capillary permeability in hypertensive patients, and this is associated with reduction of microcirculatory symptoms (Belcaro et al. 1990; De Sanctis et al. 2001). Anti-inflammatory, anti-angiogenesis and antioxidant effects of asiatic acid have also been reported previously (Huang et al. 2011; Kavitha et al. 2011; Pakdeechote et al. 2014). A study conducted using diabetic rats has reported that the ability of asiatic acid to reduce plasma glucose level might be associated with the hypolipidemic effect of asiatic acid (Ramachandran et al. 2014). Besides, asiatic acid also improves lipid profile of metabolic syndrome rats through maintaining the equilibrium between iNOS and eNOS expression (Pakdeechote et al. 2014).

Besides, asiatic acid protects against high fat diet-induced liver injury in mice through inhibiting the NF-$\kappa$B and mitogen-activated protein kinase (MAPK) pathways (Yan et al. 2014). These previous data suggest that asiatic acid possesses potential lipid lowering and anti-inflammatory effects. However, anti-atherosclerotic effects of asiatic acid and its underlying mechanisms are not well documented. Therefore, the aim of this study was to investigate the protective effects of asiatic acid on early atherogenic events, in the context of endothelial cell activation triggered by TNF-$\alpha$. We examined the *in vitro* effects of asiatic acid on TNF-$\alpha$-induced increased endothelial permeability, increased expression of adhesion molecules, monocyte adhesion and monocyte migration. In addition, the effect of asiatic acid on NF-$\kappa$B activation elicited by TNF-$\alpha$ was also explored in this study.

## Materials and methods

### *Chemicals and reagents*

Asiatic acid was purchased from ChromaDex (CA, USA) with the purity of 93.7% (Supplementary material S1), 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) was purchased from Sigma (MO, USA). Rabbit polyclonal anti-ICAM-1 antibody, mouse monoclonal anti-E-selectin, anti-VCAM-1 and anti-PECAM-1 antibodies and anti-mouse IgG HRP-conjugated were purchased from Santa Cruz Biotechnology (Texas, USA). Rabbit monoclonal anti-phospho-I$\kappa$B$\alpha$ antibody and anti-rabbit HRP-conjugated secondary antibody were purchased from Cell Signaling Technology (MA, USA). TNF-$\alpha$ was purchased from Peprotech (NJ, USA). Simvastatin and methyl thiazoyl tetrazolium (MTT) were purchased from Calbiochem (NJ, USA).

### *Cell culture*

Human aortic endothelial cells (HAECs) were purchased from American Type Cell Culture (ATCC) and maintained in endothelial cell medium (Sciencell, CA, USA) supplemented with 5% fetal bovine serum, endothelial cell growth supplement, penicillin (100 U/ml) and streptomycin (100 $\mu$g/ml). The medium was changed every 2 days until the cells were 80–90% confluent. All the experiments were performed using cells between passage 3 and 5. U937 cells, a suspension human leukemic monocyte lymphoma cell line, were purchased from ATCC and maintained in RPMI-1640 medium containing 10% Hyclone fetal bovine serum (Thermo Fisher Scientific, IL, USA). The cell density was maintained at $1 \times 10^5$ to $2 \times 10^6$ cells per ml.

For all the experiments except the cell viability assays, HAECs were pretreated with asiatic acid (10, 20, 30 and 40 $\mu$M) for 6 h before stimulated with TNF-$\alpha$ (10 ng/ml) for another 6 h.

### *Cell viability assays*

Cell viability was assessed using MTT assay as described previously (Ng et al. 2015). HAECs were treated with various concentrations of asiatic acid (10–200 $\mu$M) for 24 h. After 24 h, we added 10 $\mu$l of MTT solution (5 mg/ml in PBS) into each well and incubated for 4 h. Then, all the solution was removed and 100 $\mu$l of DMSO was added to dissolve the purple formazan salt formed. The absorbance was read at 570 nm with a reference wavelength of 650 nm. In addition, cell viability was also assessed by a fluorometric-based assay. ATP fluorometric assay kit (Biovision, CA, USA) was used to quantify the ATP levels in viable cells.