Astragaloside IV Improves Homocysteine-Induced Acute Phase Endothelial Dysfunction via Antioxidation

Li-Hong QIU, Xi-Ji XIE, and Bi-Qi ZHANG

Department of Cardiology, The First Affiliated Hospital, College of Medicine, Zhejiang University; and Department of Pharmacy, The First Affiliated Hospital, College of Medicine, Zhejiang University; Qingchun road 79#, Hangzhou 310003, P. R. China.

Astragaloside IV, the major active component extracted from Astragalus membranaceus, exerts multipotent activities under pathophysiological conditions. Hyperhomocysteinemia, an independent risk factor for cardiovascular disease, induces oxidative stress leading to endothelial dysfunction. We investigated the effect of astragaloside IV on acute phase endothelial dysfunction induced by homocysteine. In a concentration-dependent manner, endothelial dysfunction was induced by homocysteine. In organ bath experiment using rat aortic rings, treatment with astragaloside IV resulted in an improvement of the impaired endothelium-dependent vasorelaxation by homocysteine as reflected by the higher maximal vasorelaxation to acetylcholine. However, the presence of Nω-nitro-L-arginine methyl ester hydrochloride could abolish the protective effect of astragaloside IV on homocysteine-induced vasomotor dysfunction. In human umbilical vein endothelial cells culture experiment, exposure to astragaloside IV significantly ameliorated the homocysteine-induced inactivation of nitric oxide–nitric oxide synthase signal pathway via reducing oxygen species and increasing the activity of superoxide dismutase. Additionally, pretreatment with superoxide dismutase showed a similar effect to astragaloside IV on attenuation of the homocysteine-induced endothelial dysfunction. These data support the view that astragaloside IV might be advantageous in the treatment of endothelial dysfunction induced by disturbed nitric oxide–nitric oxide synthase pathway due to oxidative stress in hyperhomocysteinemia.

Key words: astragaloside IV; homocysteine; oxidation; nitric oxide; endothelial function

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease. Elevation of homocysteine (HCY) in blood plasma (>15 mmol/l), resulting from metabolic abnormalities in HCY remethylation or transsulfuration, is common in subjects with cardiovascular disease. In the last decade, insights from the animal models have established that experimental hyperhomocysteinemia produces several phenotypic effects, including endothelial dysfunction. The most common abnormality of the dysfunction observed during hyperhomocysteinemia is impaired relaxation of conduit vessels (aorta, carotid arteries, renal, or pulmonary) or impaired dilatation of micro vessels (mesenteric, cremasteric, coronary, skeletal, or cerebral arterioles) in response to the endothelium-dependent vasodilators in rat, mice, monkey, or guinea pigs, whereas no impairment was seen in response to endothelium derived NO, which then diffuses into the adjacent vascular muscle layer and causes its relaxation, may play an important role in mediating these deleterious effects on endothelial function in hyperhomocysteinemia. Furthermore, the emerging evidence suggest that hyperhomocysteinemia leads to increased vascular superoxide production through the upregulation and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and that reactive oxygen species (ROS) generated by NADPH oxidase contribute to the vascular phenotype.

Radix Astragali is the dried root of Astragalus membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao or Astragalus membranaceus (Fisch.) Bge. It has been widely used in Chinese medicine since ancient times for the improvement of immune disorders and management of cardiovascular diseases with an excellent safety. Research suggests that this herb was able to inhibit free radicals, decreases lipid peroxidation, and increases antioxidant enzymes. Many medicinally active compounds have been isolated from this plant, including polysaccharides, flavones and astragalosides. Astragalosides is the major active component extracted from the root of Astragalus membranaceus. Previous studies from our laboratory demonstrated that crude astragalosides fraction can potently protect endothelium-dependent relaxation against the acute injury from HCY through nitric oxide regulatory pathways, in which antioxidation played a key role.

Astragaloside IV (AST-IV), 3-0-beta-D-xylopyranosyl-6-o-beta-D-glucopyranosyl-cycloastragenol (Fig. 1), was a small molecular saponin. AST-IV can exert multipotent activities under pathophysiological conditions, such as anti-hypertension, positive inotropic action, anti-inflammation, and anti-infarction. Up to now, however, whether AST-IV has an exact protective effect on vessels in hyperhomocysteinemia and the mechanism are not known. The aim of the present study is to examine the effect of AST-IV on vasomotor dysfunction induced by HCY in rat aorta and explore the underlying mechanism.

![Chemical Structure of Astragaloside IV](C41H68O14, Molecular Weight: 784)
MATERIALS AND METHODS

**Materials** The AST-IV used in this study was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), with a high purity 99% by HPLC analysis. Phenylephrine (PE), acetylcholine (ACH), HCY, sodium nitroprusside (SNP), Nω-nitro-l-arginine methyl ester hydrochloride (l-NAME) and superoxide dismutase (SOD)-polyethylene were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5-(6)-Chloromethyl-2',7'-dichloro-dihydrofluorescein diacetate (CM-H2DCF-DA) was from Molecular Probes Inc. (Eugene, U.S.A.). All chemicals were of the highest purity available.

**Organ Chamber Experiment** Animals used in this study were experimentally naive male Sprague–Dawley (SD) rats obtained from Experiment Animal Center of Zhejiang Academy of Medical Sciences, China. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Rats were killed by cervical dislocation. Thoracic arterial rings were prepared according to the method described previously by Zhang et al.20) For isometric force recording, aortic rings were mounted between two stainless steel hooks and suspended in the organ bath containing Krebs’ buffer, composed of (mM): NaCl, 118; KCl, 4.7; MgSO4·7H2O, 1.2; KH2PO4, 1.2; CaCl2, 2.5; NaHCO3, 25; and glucose, 11; at 37 °C bubbled with 95% O2 and 5% CO2 (pH 7.4). The tension of the aortic ring was monitored by a force transducer (Nanjing Medease Science and Technology Co., Ltd., China), connected to MedLab 5.0v, a computer-based data acquisition system (Nanjing Medease Science and Technology Co., Ltd., China). After equilibrium for 60 min at 2.0 g resting tension, then rings were challenged with KCl (6.0 mol/l). The ACH-induced maximal relaxation (Emax) in aortic rings was calculated as a percentage of the contraction in response to PE (1 μmol/l). The half maximum effective concentration (EC50) was designated as the concentration of ACH that induced 50% of maximum relaxation from the contraction elicited by PE (1 μmol/l). These values were determined by nonlinear regression of the log concentration–response curves. Sensitivity was expressed as pD2 (−log EC50). All values are expressed as mean±S.E.M. Statistical analysis was performed with one-way ANOVA followed by Newman–Keuls test. Differences were accepted as statistically significant at p values <0.05 (Graph Pad Prism).

**Clinical and Biological Products (Beijing, China), with a high purity 99% by HPLC analysis. Phenylephrine (PE), acetylcholine (ACH), HCY, sodium nitroprusside (SNP), Nω-nitro-l-arginine methyl ester hydrochloride (l-NAME) and superoxide dismutase (SOD)-polyethylene were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 5-(6)-Chloromethyl-2',7'-dichloro-dihydrofluorescein diacetate (CM-H2DCF-DA) was from Molecular Probes Inc. (Eugene, U.S.A.). All chemicals were of the highest purity available.

**Organ Chamber Experiment** Animals used in this study were experimentally naive male Sprague–Dawley (SD) rats obtained from Experiment Animal Center of Zhejiang Academy of Medical Sciences, China. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Rats were killed by cervical dislocation. Thoracic arterial rings were prepared according to the method described previously by Zhang et al.20) For isometric force recording, aortic rings were mounted between two stainless steel hooks and suspended in the organ bath containing Krebs’ buffer, composed of (mM): NaCl, 118; KCl, 4.7; MgSO4·7H2O, 1.2; KH2PO4, 1.2; CaCl2, 2.5; NaHCO3, 25; and glucose, 11; at 37 °C bubbled with 95% O2 and 5% CO2 (pH 7.4). The tension of the aortic ring was monitored by a force transducer (Nanjing Medease Science and Technology Co., Ltd., China), connected to MedLab 5.0v, a computer-based data acquisition system (Nanjing Medease Science and Technology Co., Ltd., China). After equilibrium for 60 min at 2.0 g resting tension, then rings were challenged with KCl (6.0 mol/l). The ACH-induced maximal relaxation (Emax) in aortic rings was calculated as a percentage of the contraction in response to PE (1 μmol/l). The half maximum effective concentration (EC50) was designated as the concentration of ACH that induced 50% of maximum relaxation from the contraction elicited by PE (1 μmol/l). These values were determined by nonlinear regression of the log concentration–response curves. Sensitivity was expressed as pD2 (−log EC50). All values are expressed as mean±S.E.M. Statistical analysis was performed with one-way ANOVA followed by Newman–Keuls test. Differences were accepted as statistically significant at p values <0.05 (Graph Pad Prism).

**RESULTS**

**Organ Chamber Experiment** In each group, a sustained vascular contraction with a peak tension of about 2.65±0.31 g was induced by PE (1 μmol/l). In any group, no difference in relaxation responses to ACH (10 μmol/l) in aortic rings was observed at the initial experiment.

Compared with the control group (Emax: 98.37±0.65%, pD2: 6.88±0.05), HCY (0.1—3 mmol/l) treatment inhibited...
endothelium-dependent relaxation evoked by ACH, and the $E_{max}$ fell to 84.90 ± 1.01%, 76.99 ± 1.56%, 65.20 ± 1.50%, and 51.18 ± 1.89%, with a reduction of pD$_2$ (6.74 ± 0.06, 6.62 ± 0.05, 6.53 ± 0.05, and 6.50 ± 0.05). There was no significant difference of pD$_2$ was found between 1 mmol/l and 3 mmol/l HCY group, although $E_{max}$ exerted a further reduction (Fig. 2). Moreover, HCY (0.1—3 mmol/l) failed to deteriorate the endothelium-independent relaxation to SNP (data not shown). Therefore, 1 mmol/l HCY treatment-induced inhibition of endothelium-dependent relaxation to ACH was used in the subsequent experiment as a control.

Treatment with AST-IV (50 or 100 μg/ml) was able to improve the inhibition of endothelium-dependent relaxation induced by HCY (1 mmol/l). $E_{max}$ and pD$_2$ were shown as (74.67 ± 2.10% or 81.73 ± 1.87% versus 65.20 ± 1.50%, $p<0.05$) and (6.78 ± 0.06 or 6.87 ± 0.06 versus 6.53 ± 0.05, $p<0.05$), respectively. Additionally, due to the presence of L-NAME (100 μmol/l), the inhibitor of nitric oxide synthase (NOS), AST-IV (100 μg/ml) failed to show any ameliorating effect on endothelium-dependent vasomotor response caused by HCY (1 mmol/l) (Fig. 3).

To confirm the oxidative effect of HCY on endothelium-dependent relaxation, SOD was used. With the increase of the concentration of SOD in organ bath (0.6, 1.2, and 1.8 KU/l), a more significant effect was observed ($E_{max}$: 70.59 ± 1.72%, 82.18 ± 1.26%, and 93.51 ± 1.30% versus 65.20 ± 1.50%; pD$_2$: 6.68 ± 0.06, 6.91 ± 0.06, and 9.94 ± 0.06 versus 6.53 ± 0.05) and the inhibitory effect of HCY was almost completely abolished. In an analogous manner, combined treatment of the rings with AST-IV (100 μg/ml) and SOD (1.2 KU/l) totally reversed the HCY-induced impairment of endothelium-dependent relaxation ($E_{max}$: 95.10 ± 2.04%; pD$_2$: 6.91 ± 0.06), which was very similar to the effect of 1.8 KU/l SOD group (Fig. 4).

Cellular Experiments Incubation of HUVECs with HCY (0.1 or 1 mmol/l) for 60 min resulted in the reduction both of NO content and NOS activity. Treatment with AST-IV (50 and 100 μg/ml) was able to attenuate the inhibition both of NO content and NOS activity. Furthermore, SOD (1.8 KU/l) exerted more significant ameliorating effects on these parameters than AST-IV (100 μg/ml). Meanwhile, combined treatment with SOD (1.2 KU/l) and AST-IV (100 μg/ml) induced a similar effect with SOD (1.8 KU/l) (Figs. 5, 6).

Compared to the control group, treatment with HCY (1 mmol/l) for 60 min significantly increased the ROS level and decreased the SOD activity in HUVECs. AST-IV (10, 50, and 100 μg/ml) markedly prevented this accumulation of ROS and reduction of SOD activity. However, AST-IV showed a lower potency than SOD (Figs. 7, 8).
Redox reactions resulting in increased vascular levels of ROS may play an important role in mediating endothelial dysfunction, particularly the loss of endothelium-derived NO, during hyperhomocysteinemia. The present study indicated that AST-IV significantly increased intracellular ROS lev-
els and decreased the SOD activity in HUVECs, which was consistent with the reported by Lin et al. Treatment with SOD, an antioxidant enzyme, reversed the HCY-impaired endothelium-dependent vasomotor responses. Other researchers have suggested that SOD showed similar protective effects on injury aorta, pulmonary artery, or skeletal muscle arterioles.

During these years, natural products with antioxidant activity have drawn the most attention. Astragalus membranaceus is widely used for prevention of ROS-mediated injury in pathological situation through its antioxidant properties. In an in vitro study, it was demonstrated by electron paramagnetic resonance imaging technique that Astragalus membranaceus can potently inhibit ROS produced by dimethyl sulfoxide system and scavenge over 90% of ROS. Recently, the emerging experiments reported that a herbal formulation, comprising Astragalus membranaceus, improved the pancreatic beta cell function via the antioxidative effect. Furthermore, total saponins extracted from Astragalus membranaceus had the effects on enhancing free radical removal and decreasing lipid peroxidation, thus preventing the calcium overload in isoproterenol-treated cardiomyocytes. According to our previous study, it has been shown that total saponins isolated from Astragalus membranaceus ameliorated endothelium-dependent relaxation induced by HCY via scavenging free radical species.

Astragalus membranaceus contains a series of cycloartane triterpene glycosides denoted astragalosides I—VII (saponins), which are based on the aglycone cycloastragenol and contain from one to three sugars attached at the 3-, 6-, and 25-positions. AST-IV is regarded as the main criterion of quality control of Astragalus membranaceus in the Pharmacopoeia of the People’s Republic of China. There is emerging evidence that AST-IV has the biological property of eliminating ROS, due to which it protects cardiomyocytes from oxidative stress-mediated injury under hypoxic conditions, delays the aging effect in rats treated by hydrocortisone, and protects coxsackievirus B3-induced murine myotations, delays the aging effect in rats treated by hydrocortisone, from oxidative stress-mediated injury under hypoxic conditions. Due to which it protects cardiomyocytes from ROS, due to which it protects cardiomyocytes from oxidative stress-mediated injury under hypoxic conditions, delays the aging effect in rats treated by hydrocortisone, and protects coxsackievirus B3-induced murine myotations, delays the aging effect in rats treated by hydrocortisone, from oxidative stress-mediated injury under hypoxic conditions.

In summary, it was demonstrated for the first time that AST-IV has the ability to regulate NO pathway impaired by HCY through antioxidant defense. In light of these findings, it could lead to the development of a new therapy for the vascular lesion in the pathological condition characterized by elevation of HCY in blood plasma.

Acknowledgements This study supported by grants from Province Administration of Traditional Chinese Medicine, Zhejiang, P.R. China (Project Number: 2009CA055).

REFERENCES


