Asiatic Acid Alleviates Fibrosis but not Cardiac Hypertrophy in the Left Ventricle of L-NAME-induced Hypertensive Rats

เอเซียติก แอซิด ลดไฟโบรซีสแต่ไม่มีผลต่อภาวะหัวใจโตในหัวใจห้องล่างซ้าย ในหนูขาวความดันเลือดสูงที่ถูกเหนี่ยวนำด้วยสารแอลเนม

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ABSTRACT

This study aimed to investigate whether asiatic acid (AA) could alleviate left ventricular (LV) hypertrophy and myocardial fibrosis in N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced hypertensive rats. Hypertension was induced by administration of L-NAME (40 mg/kg/day) in drinking water for 5 weeks. Treatment with AA (10 mg/kg/day) for the last 2 weeks markedly reduced blood pressure and alleviated myocardial fibrosis ($P < 0.05$) in L-NAME-treated rats. These effects of AA were associated with elevated plasma nitric oxide metabolites (NOx) levels, together with upregulation of eNOS expression and decreased malondialdehyde (MDA) concentration in the heart. This study suggests that AA reduced blood pressure and myocardial fibrosis in LNAME-induced hypertensive rats, the mechanism might be related to an increase in nitric oxide (NO) levels and a decrease of oxidative stress status.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาสารเอเซียติก แอซิด สามารถลดภาวะหัวใจห้องล่างซ้ายโตและการเกิดไฟโบรซีสของกล้ามเนื้อหัวใจ ในหนูขาวความดันเลือดสูงที่ถูกเหนี่ยวนำด้วยสารแอลเนมได้หรือไม่ ภาวะความดันเลือดสูงถูกเหนี่ยวนำด้วยสารแอลเนม (40 มก./กก./วัน) ผสมในน้ำดื่มเป็นเวลา 5 สัปดาห์ โดยพบว่าการให้เอเซียติก แอซิด (10 มก./กก./วัน) ในช่วง 2 สัปดาห์สุดท้าย สามารถลดระดับความดันเลือดและและลดการเกิดไฟโบรซีสของกล้ามเนื้อหัวใจ ($P < 0.05$) ในหนูทดลองที่ได้รับสารแอลเนม โดยผลดังกล่าวมีความสัมพันธ์กับการเพิ่ม plasma NOx พร้อมทั้งการเพิ่ม eNOS expression และการลดระดับของ MDA ในเนื้อเยื่อหัวใจ การศึกษาครั้งนี้แสดงให้เห็นว่า เอเซียติก แอซิด สามารถลดภาวะความดันเลือดสูงและลดการเกิดไฟโบรซีสของกล้ามเนื้อหัวใจในหนูขาวความดันเลือดสูงที่ถูกเหนี่ยวนำด้วยสารแอลเนม โดยกลไกอาจเกี่ยวข้องกับการเพิ่มของ NO และการลดภาวะเครียดออกซิเดชัน

Key Words: Asiatic acid, Hypertension, Oxidative stress

คำสำคัญ: เอเซียติก แอซิด ภาวะความดันเลือดสูง ภาวะเครียดออกซิเดชัน

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Introduction

Left ventricular (LV) hypertrophy is an adaptive reaction to increased haemodynamic load. It represents an independent risk factor of increased cardiovascular morbidity and mortality (Simko, 2002). Chronic administration of N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), a nonspecific inhibitor of all three nitric oxide synthase (NOS), has been reported to induce the development of systemic arterial hypertension (Baylis et al., 1992; Krier and Romero, 1998). In addition, the increase in blood pressure during nitric oxide (NO) deficiency is associated with LV hypertrophy and fibrosis (Paulis et al., 2008).

Asiatic acid (AA) is a triterpenoid compound derived from the medicinal plant, *Centella asiatica*. The pharmacological activities of AA such as antioxidant (Wei et al., 2013), antihyperlipidemic (Pakdeechote et al., 2014), antidiabetic, (Ramachandran et al., 2013) and anti-inflammatory (Huang et al., 2011) properties have been demonstrated. In addition, our previous study found that AA reduced blood pressure with an enhancement of NO bioavailability in L-NAME-treated rats (Bunbupha et al., 2014). Although a wide range of potentially therapeutic effect of AA have been reported, little is known about the effect of AA on LV hypertrophy and fibrosis in chronic nitric oxide-deficient hypertensive rats.

Objectives of the study

This study aimed to evaluate whether AA could reduce LV hypertrophy and fibrosis in rats with hypertension induced by L-NAME.

Methodology

Animal and Experimental protocols

Male Sprague-Dawley rats (220-240 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. Rats were maintained in an air-conditioned room (25 ± 2 °C) with a 12 h dark-light cycle at Northeast Laboratory Animal Center. All procedures complied with the standards for the care and use of experimental animals and were approved by Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (AEKKU 37/2555).

After 1 week of acclimatization, the animals were randomly divided into 2 main groups. Group 1 is a normal control group which received tap water for 5 weeks. Group 2 is an L-NAME-treated group which received L-NAME (40 mg/kg/day) in their drinking water for 5 weeks to induce hypertension. The animals in all experimental groups were fed with a standard chow diet (Chareon Pokapan Co. Ltd., Thailand). After 3 weeks of study, hypertensive rats were divided in to 2 groups (n = 6/group); hypertensive rats treated with vehicle (propylene glycol) and hypertensive rats received AA (10 mg/kg/day) for the last 2 weeks.

Blood pressure measurement

Systolic blood pressure (SBP) of animals was measured weekly using non-invasive tail-cuff plethysmography (IITC/Life Science Instrument model 229 and model 179 amplifiers, Woodland Hills, CA, USA). In brief, conscious rats were placed in a restrainer and allowed to calm prior to blood pressure measurement. The rat tail was placed inside the tail cuff, and the cuff was automatically inflated and released. For each rat, blood pressure was recorded as the mean value from the three measurements with 15-min intervals.

Heart weights, tissue sampling and blood plasma isolation

At the end of study, the animals were anesthetized by peritoneal injection of pentobarbital-sodium (60 mg/kg). Body weights (BW) were recorded and blood samples were collected from the abdominal aorta in EDTA tubes. After blood sampling the animals were sacrificed by over dosage of the anesthetic drug. Heart wet weight and left ventricular wet weight (LVW) were measured, and LVW to BW ratio (LVW/BW) were calculated. Samples of the left ventricle were used for the determination of eNOS expression, and histological study.

Assay of nitric oxide metabolites (NOx)

NOx assay was performed following the previous study (Luangaram et al., 2007). Briefly, plasma samples were deproteinized by ultrafiltration using centrifugal concentrators (Pall Corp., Ann Arbor,MI, USA). The supernatant was mixed with 1.2 μmol/L NADPH, 4 mmol/L glucose-6-phosphate disodium, 1.28 U/mL glucose-6-phosphate dehydrogenase, and 0.2 U/mL nitrate reductase and then incubated at 30°C for 30 min. The mixture was then reacted with Griess solution (4% sulfanilamide in 0.3% NED) for 15min. The absorbance of samples at 540nm was measured on a microplate reader (Tecan GmbH., Grodig, Austria).

Assay of malondialdehyde (MDA)

The concentration of MDA in plasma, aortic and heart tissues were measured as TBA reactive substances by a spectrophotometric method as previously described (Draper et al., 1993). In brief, 150-μL plasma samples were reacted with 10% TCA, 5 mmol/L EDTA, 8% SDS, and 0.5 μg/mL BHT. The mixture was incubated for 10 min at room temperature, then 0.6% TBA was added, and the mixture was boiled in a water bath for 30 min. After cooling to room temperature, the mixture was centrifuged at 10,000 g for 5 min. The absorbance of the supernatant was measured at 532 nm by a spectrophotometer (Amersham Bioscience, Arlington, MA, USA). A standard curve was generated at different concentrations from 0.3 to 10 μmol/L using 1,1,3,3-tetraethoxypropane. The MDA concentration was normalized against the protein concentration. Protein was determined by the Bradford dye binding method.

Histology and morphometry

The tissues were fixed 24 hours in 10% formalin, routinely processed in paraffin and 5 μm thick slides from the midventricular surface, either to the base or to the apex, were stained with Hematoxylin and Eosin (H&E) and picro-sirius red. Morphometric evaluations of LV cross section area and fibrosis were evaluated with Image-J NIH image analysis software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

Protein eNOS expression levels were determined in heart tissue homogenates following a previously described Western blot method (Mukai and Sato, 2009), with some modifications. Homogenates were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel electrophoresis system. The proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane, blocked with 5% skimmed milk in phosphate buffer saline with 0.1% Tween-20 (PBST) for 2 hours at room temperature before overnight incubation at 4°C with mouse monoclonal antibodies to eNOS (BD Biosciences, San Jose, CA, USA) and mouse monoclonal antibodies to

GAPDH (Santa Cruz Biotechnology). The membranes were washed with PBST and then incubated for 2 hours at room temperature with horseradish peroxidase conjugated secondary antibody. The blots were developed in Amersham™ ECL™ Prime solution (Amersham Biosciences Corp., Piscataway, NJ, USA) and densitometric analysis was performed using an ImageQuant™400 imager (GE Healthcare Lifescience, Piscataway, NJ, USA). The intensity of the bands was normalized to that of GAPDH, and data were expressed as a percentage of the values determined in control group from the same gel.

Statistical analysis

Data are presented as means ± standard error of mean (SEM). Statistical comparisons among groups were made using one-way analysis of variance (ANOVA) with a Student Newman–Keul's test. Statistical significance was determined at a level of $P < 0.05$.

Results

Effects of AA on blood pressure

At the beginning of the study, there were no significant differences in average baseline values of SBP among all groups of rats (Figure 1). In the control group, the SBP was not altered during the experiment. Administration of L-NAME for 5 weeks induced a progressive increase in SBP (95% after 5 weeks of treatment as compared with the SBP of the control rats; $P < 0.001$). Treatment with AA at the fourth and fifth week significantly reduced SBP in hypertensive rats as compared with hypertensive rats receiving vehicle (11% at 4th week; $P < 0.05$ and 19% at 5th week; $P < 0.05$).

Effect of AA on cardiac mass indexes

After the 5 weeks of experiment, the BW was not significantly different among the groups (Table 1). Chronic administration of L-NAME caused an increase in the heart weight and LVW/BW ratio ($P < 0.05$). However, administration with AA did not significantly affect on heart weight and LVW/BW.

Effect of AA on cardiac morphometry

Histomorphometric analysis showed that chronic L-NAME treatment significantly increased cross section area and fibrosis of LV when compared to those of the normal control group ($P < 0.05$). Administration of AA significantly alleviated the enlargement of LV fibrosis in L-NAME-induced hypertensive rats (Figure 2; $P < 0.05$). However, treatment with AA in L-NAME-induced hypertensive rats had no effect on cross sectional area (Table 1).

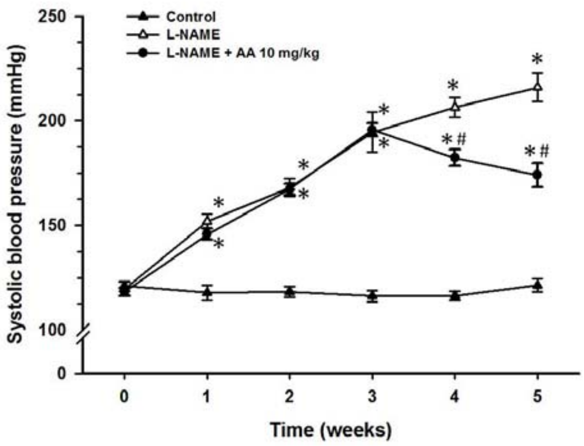

Figure 1 Effect of AA on SBP during L-NAME administration for 5 weeks in hypertensive rats. Results are expressed as mean ± SEM. *$P < 0.05$ vs. normal control group, $^{\#}P < 0.05$ vs. L-NAME group ($n = 6$/group).

Table 1 Effect of AA on cardiac mass indexes and morphometry

	BW (g)	Heart weight (mg)	LVW/BW (mg/g)	LV cross sectional area (mm^2)
Control	410.3 ± 4.8	1,378.4 ± 27.2	2.3 ± 0.08	51.2 ± 1.6
L-NAME	416.8 ± 12.4	1,546.3 ± 28.8*	3.0 ± 0.09*	59.3 ± 1.0*
L-NAME + AA	414.5 ± 10.1	1,522.4 ± 30.4	2.9 ± 0.15	57.7 ± 1.2

Results are expressed as mean ± SEM. *P < 0.05 vs. control group (n = 5/group).

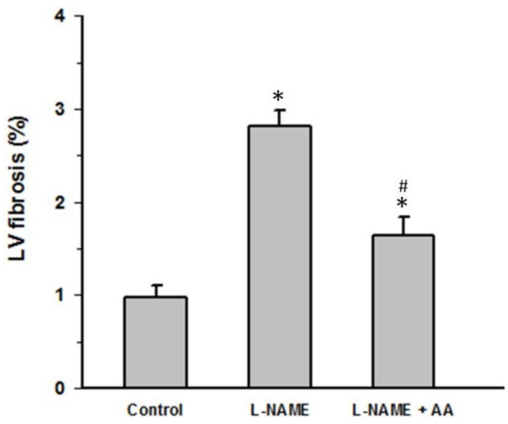

Figure 2 Effect of AA on LV fibrosis in hypertensive rats. Results are expressed as mean ± SEM. *P < 0.05 vs. normal control group, # P< 0.05 vs. L-NAME group (n = 5/group).

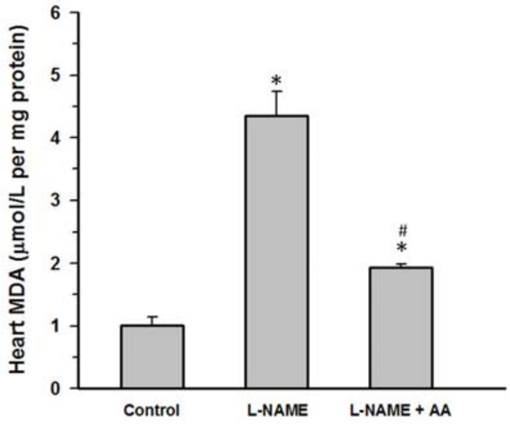

Figure 3 Effect of AA on MDA concentration of heart tissue in hypertensive rats. Results are expressed as mean ± SEM. *P < 0.05 vs. normal control group, #P < 0.05 vs. L-NAME group (n = 5/group).

Effect of AA on MDA concentration

MDA levels in heart tissue were significantly higher in L-NAME-induced hypertensive rats (4.4 ± 0.39 µmol/L/mg protein; Figure 3) than those of normal rats (1.0 ± 0.12 µmol/L/mg protein; P < 0.05). However, an increase in heart tissue MDA levels in L-NAME-induced hypertensive rats was attenuated by AA supplementation (1.9 ± 0.06 µmol/L/mg protein; P < 0.05).

Effect of AA on plasma NOx levels and eNOS protein expression in heart tissues

In L-NAME-treated rats, plasma NOx concentrations were significantly reduced (2.1 ± 0.3 µmol/L; Figure 4A) compared with those of the normal control group (8.5 ± 0.8 µmol/L; P < 0.05). Moreover, a reduction of plasma NOx was consistent with downregulation of eNOS protein expression in heart tissues of hypertensive rats (P < 0.05; Figure 4B). Oral supplementation with AA significantly improved the concentration of plasma NOx (4.6 ± 0.6

µmol/L; $P < 0.05$) and restored heart eNOS protein expression ($P < 0.05$) in hypertensive rats.

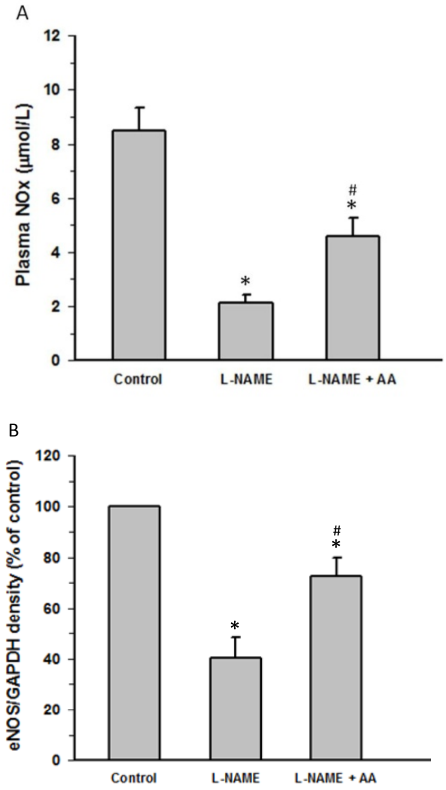

Figure 4 Effect of AA on (A) plasma NOx and (B) eNOS expression in hypertensive rats. Results are expressed as mean ± SEM. $^{*}P < 0.05$ vs. normal control group, $^{\#}P < 0.05$ vs. L-NAME group (plasma NOx n=5/group; eNOS expression n=3/group).

Discussion and Conclusions

The present study demonstrates the effects of AA on blood pressure and cardiac wall changes in L-NAME-induced hypertension. Chronic L-NAME treatment caused an increase in blood pressure, LVW/BW ratio, LV hypertrophy as well as myocardial fibrosis. These alterations were associated with decreased plasma NOx concentrations, downregulation of eNOS expression in the heart tissues, and increased levels of oxidative stress markers. Treatment of L-NAME-induced hypertensive rats with AA improved plasma NOx concentrations by restoring eNOS expression and reducing MDA in heart tissue. This, in turn, reduced blood pressure and ameliorated LV fibrosis during NO deficient rats.

Our results confirm previous studies that chronic inhibition of NO synthesis with L-NAME induces a systemic arterial hypertension (Baylis et al., 1992; Krier and Romero, 1998). We found that treatment of L-NAME-induced hypertensive rats with AA lessened the increase in blood pressure. The reduction of blood pressure in this experiment might be related to enhancing of NO bioavailability (Bunbupha et al., 2014).

L-NAME-induced hypertension in rats is characterized by an increased in blood pressure and associated with cardiac fibrosis and hypertrophy (Bernatova et al., 2000; Pechanova et al., 2004). This present study revealed increases in heart weight, LVW/BW ratio, LV cross sectional area, and cardiac fibrosis in L-NAME-treated rats. AA alleviated LV fibrosis but had no effect on LV hypertrophy in hypertensive rats. The plausible explanation is that AA decreased LV fibrosis might be related with the restoring of eNOS expression, NO levels, and the reduction of cardiac oxidative stress. This is supported by Kumar and coworkers (2014). They found that upregulation of eNOS mRNA expression with restoring NOx levels can inhibit of cardiac wall remodeling in rats with L-NAME-induced hypertension (Kumar et al., 2014). In addition, there is evidence to support the association between oxidative stress and cardiac fibrosis. Cardiac oxidative stress promotes the development of cardiac fibrosis by upregulating TGF-beta1 expression, which subsequently enhances cardiac collagen synthesis and suppresses collagen degradation in hypertensive rats (Zhao et al., 2008).

In conclusion, AA is able to attenuate the increasing in blood pressure together with alleviates cardiac fibrosis but not cardiac hypertrophy in L-NAME-induced hypertensive rats. This might be related to an increase in NO levels and a decrease of oxidative stress status.

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