

Original Research Article

Anhuienoside C ameliorates atherosclerosis in rats via regulation of the NFκB/eNOS/NO signaling pathway

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Abstract

Purpose: To investigate the protective effect of anhuienoside C (AC) against high cholesterol diet-induced atherosclerosis in a rat model.

Methods: Atherosclerosis was induced in rats by administration of high fat diet for 8 weeks, and AC (20 and 40 mg/kg) was administered orally. The effect of AC was determined by assessing serum lipid profiles and mediators of inflammation, as well as oxidative stress parameters in aortic tissue using enzyme-linked immunosorbent assay (ELISA). Western blot assay and reverse transcription polymerase chain reaction (RT-PCR) were used for the evaluation of protein expressions.

Results: Serum levels of total cholesterol (TC), triglycerides (TGs), high density lipoprotein (HDL), low density lipoprotein (LDLP), and IL-1β, IL-18, TNF-α and NF-κB were significantly reduced in AC-treated group, relative to atherosclerotic rats ($p < 0.01$). Moreover, parameters of oxidative stress were attenuated in the aortic tissues of AC-treated group, when compared with atherosclerotic rats. There was significant increase in eNOS expression, and marked decrease in the expressions of MAPK and NF-κB protein in the aortic tissue homogenate of AC treated group, relative to atherosclerotic group ($p < 0.01$). Treatment with AC attenuated the mRNA expressions of ICAM-1, VCAM-1 and MCP-1 in aortic tissue of the atherosclerotic rats.

Conclusion: These results reveal that AC prevents atherosclerosis in rats by modulating the NFκB/eNOS/NO signaling pathway, and thus, can thus potentially be developed as anti-atherosclerotic agent

Keywords: Anhuienoside C, Atherosclerosis, Inflammation, Oxidative stress, Cholesterol

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INTRODUCTION

Atherosclerosis is a coronary artery disease in which the diameter of arteries become reduced due to deposition of cholesterol and blood cells [1]. In atherosclerosis, plaque formation in the

arteries induces ischemic heart diseases such as acute coronary syndrome, stroke and heart attack [1]. Factors that predispose to atherosclerosis include diabetes, hypertension and dyslipidemia [2]. In patients suffering from dyslipidemia, there is elevation in serum

concentration of LDL, resulting in some pathological changes such as inflammation, oxidative stress and endothelium injury [3]. Moreover, atherosclerosis may develop due to inflammatory responses associated with vascular endothelial dysfunction. Levels of NO which are regulated by eNOS also contribute to the pathogenesis of atherosclerosis [4]. Expression of inflammatory molecules such as MCP-1, VCAM-1 and ICAM-1 are enhanced in atherogenesis due to endothelial dysfunction [5]. Studies have revealed that the expressions of VCAM-1 and ICAM-1 are up-regulated in atherosclerosis due to impairment of NO [6]. Moreover, in endothelial cells, expression of MCP-1 is enhanced due to inhibition of NO synthesis. Several molecules of natural origin have shown beneficial effects in the management of atherosclerosis. Anhuienoside C (AC) isolated from the *Anemone flaccida* Fr. Schmidt belongs to family Ranunculaceae [7]. In China, *Anemone flaccida* Fr. Schmidt, popularly called *Di Wu* is used traditionally in for the treatment of chronic disorders. It is reported to possess antiarthritic and anti-oxidant properties, as well as NO-reducing effect in macrophages [8]. The present investigation was carried out to determine the beneficial effect of AC on high cholesterol diet-induced atherosclerosis in rats.

EXPERIMENTAL

Animals

Male Sprague-Dawley rats weighing 150-200 g were kept in 12-h light/12-h dark cycle under standard conditions (60 ± 5 % humidity and temperature of 24 ± 3 °C). The study protocols were approved by institutional animal ethical committee of The First Affiliated Hospital of Xi'an Jiaotong University, China (no. IAEC/FAH-XJU/2017/09). The Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines was applied in the handling of the animals used in this study [9].

Treatments

Atherosclerosis was induced in the rats by administration of high fat diet as per previously reported. High cholesterol diet-induced atherosclerosis was produced by administering 70 U/kg of vitamin D₃ for the period of 3 consecutive days. Thereafter, high-cholesterol diet (HCD, a combination of 80.3 % normal diet with 4 % refined sugar, 0.7 % propylthiouracil, 1.5 % sodium cholate, 4.5 % cholesterol, and 11 % animal oil) was administered to all rats other than those in the normal control group, for the

duration of 8 weeks. The rats were divided into four different groups: normal, atherosclerosis, AC (20 mg/kg) and AC (40 mg/kg) groups. The AC doses were given p.o. for 8 weeks. Biochemical analyses were carried out on blood collected through retro-orbital bleeding. At the end of the protocols, the rats were sacrificed using cervical dislocation, and aortic tissue was isolated from each rat.

Assessment of biochemical parameters

Serum was separated from the blood by centrifuging it at 2000 rpm for 10 min at room temperature. Serum concentrations of low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TG) and total cholesterol (TC) were determined as per the direction given by the manufacturer of their commercial kits. Serum levels of the inflammatory mediators NF-κB, IL-1β, IL-18 and TNF-α in all groups were estimated using ELISA kits as per the kit directions.

Evaluation of oxidative stress

Levels of malondialdehyde (MDA), nitric oxide (NO) and reduced glutathione (GSH), and activities of superoxide dismutase (SOD) and inducible nitric oxide synthase iNOS were estimated in the aortic tissues using ELISA kits, as per manufacturer's protocols.

Western blot assay

Total protein was extracted from isolated aortic tissues after treating them with NP40 protein lysis buffer, and DC protein assay was used for the estimation of total protein concentration in each group. Sodium dodecyl sulfate (SDS)-polyacrylamide gel (10 %) electrophoresis was used for the separation of isolated protein. The protein bands were then transferred to polyvinylidene difluoride membrane. The membrane was treated with 5 % fresh non-fat dry milk to block non-specific binding of the blot. This was followed by incubation of the membrane overnight at 4 °C with primary antibodies for NF-κB, p65, eNOS, p38 MAPK and GAPDH. Thereafter, the membrane was incubated with secondary antibody for 60 min at room temperature. Chemiluminescence was used to enhance the blot, while ImageLab software was used to perform the densitometric analysis of the blots.

Reverse transcription polymerase chain reaction (RT-PCR)

Trizol reagent was used to isolate total RNA

from aortic tissue. The RNA was then reverse-transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit. The primers (indicated below) were used to determine the gene expression with RT 2 SYBR Green Master, with Quantitative SYBR Green PCR assays. The program used in all samples was 98 °C for 2 min, and then 25 to 40 cycles of 98 °C for 10 sec; 55 °C for 5 sec, and 72 °C for 20 sec. The mRNA expression levels were calculated according to relative standard curves. The curves were generated by plotting the quantification cycle (Cq) against the log amount of total cDNA added to the reaction. The relative target gene expression levels were determined using the $2^{-\Delta\Delta Cq}$ method.

Primer	Forward
	Reverse
ICAM-1	5' CAGTGA CCATCTACA GCTTTCCGG 3' 5'GCTGCTACCACAGTGAT GACAA3'
VCAM-1	5' CCC TTG ACC GGC TGG AGA TT 3' 5' TGG GGG CAA CAT TGA CAT AAA GTG 3'
MCP-1	5' CCC CAG TCA CCT GCT GTT AT 3' 5' CCA CAA TGG TCT TGA AGA TCA C 3'
GAPDH	5' ACG GAT TTG GTC GTA TTG GG 3' 5' TGA TTT TGG AGGGAT CTC GC 3'

Histopathological assessment of aortic arch

Isolated aortic arch was fixed by seeding it in 10 % formalin solution. The tissue was sectioned into 6-µm thickness and stained with hematoxylin and eosin (H&E). Morphological assessment was done by calculating the ratio of thickness between intima and intima plus media using a trinocular microscope.

Statistical analysis

All data are shown as mean ± standard error mean (SEM) (n = 8). All results were analysed with one-way ANOVA and Dunnett's post hoc

test (Gradpad prism 6.1., CA, USA). Values of $p < 0.05$ were considered significant.

RESULTS

Effect of AC on lipid profile

Table 1 shows the effect of AC on serum TGs, TC, LDL and HDL in the high fat diet induced-atherosclerotic rats. The serum levels of TGs, TC, LDL and HDL in the atherosclerosis group were elevated, when compared to normal control group of rats. However, treatment with AC attenuated the altered levels of serum lipids in the atherosclerotic rats.

Effect of AC on mediators of inflammation

The effect of AC on serum levels of inflammatory mediators in atherosclerotic rats is shown in Figure 1. Serum concentrations of IL-1β, IL-18, TNF-α and NF-κB were enhanced in atherosclerosis group, when compared to the normal control group of rats. However, AC treatment resulted in dose-dependent decreases in the serum concentrations of IL-1β, IL-18, TNF-α and NF-κB, relative to the atherosclerosis group.

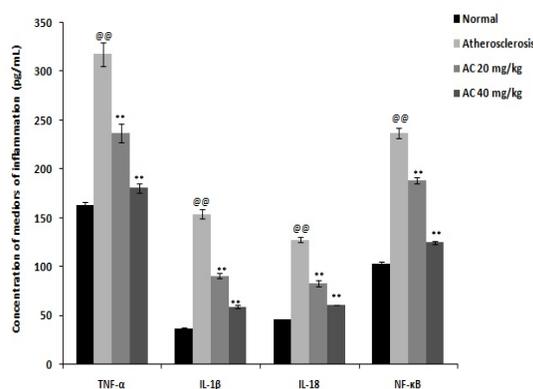


Figure 1: Effect of AC on serum levels of mediators of inflammation in artherosclerotic rats. Data are mean ± SEM (n = 8); @@ $p < 0.01$, compared to normal group; * $p < 0.05$, ** $p < 0.01$, compared to atherosclerosis group

Table 1: Effect of AC on serum lipid profile of high fat diet-induced artherosclerotic rats

Group	HDL (mmol/L)	LDL (mmol/L)	TGs (mmol/L)	TC (mmol/L)
Normal	0.72±0.03	0.69±0.02	0.87±0.03	1.47±0.07
Atherosclerosis	0.34±0.01@@	7.64±0.26@@	6.14±0.18@@	7.49±0.21@@
AC (20 mg/kg)	0.47±0.02**	4.98±0.17*	4.82±0.15*	5.13±0.14**
AC (40 mg/kg)	0.64±0.04**	1.93±0.12**	2.91±0.13**	3.21±0.09**

Data are mean ± SEM (n = 8); @@ $p < 0.01$, relative to normal group; * $p < 0.05$, ** $p < 0.01$, compared to atherosclerosis group

Table 2: Effect of AC on markers of oxidative stress in tissue homogenate of atherosclerotic rats

Group	MDA (nmol/mg protein)	GSH (nmol/mg protein)	SOD (U/mg protein)	NO (nmol/mg protein)	iNOS (U/mg protein)
Normal	1.16±0.02	9.62±0.26	2.36±0.04	1.26±0.05	1.28±0.07
Atherosclerosis	7.41±0.06@@	3.74±0.09@@	1.14±0.01@@	2.13±0.07@@	2.14±0.16@@
AC (20 mg/kg)	4.32±0.03**	4.91±0.14**	1.49±0.03**	1.79±0.09**	1.82±0.09*
AC (40 mg/kg)	2.31±0.02**	6.32±0.18**	1.92±0.06**	1.43±0.06**	1.37±0.05**

Mean ± SEM (n = 8); @@p < 0.01, compared to normal group; *p < 0.05, **p < 0.01, compared to atherosclerosis group

Effect of AC on parameters of oxidative stress

Table 2 shows levels of markers of oxidative stress in the aortic tissue homogenate of rats treated with AC. The MDA and NO levels were enhanced, while GSH level was reduced in the tissue homogenate of atherosclerosis group, when compared with the normal group of rats. Moreover, SOD activity was reduced, while the activity of iNOS was enhanced in the aortic tissue homogenate of atherosclerosis group, relative to the normal control group of rats. However, AC treatment ameliorated the altered levels of MDA, NO and GSH, as well as activities of SOD and iNOS in the aortic tissue homogenate of atherosclerotic group of rats.

Effect of AC on protein expressions of NF-κB, eNOS and MAPK

Figure 2 shows the effect of AC on the protein expressions of NF-κB, eNOS and p38 MAPK in the aortic tissue homogenates of atherosclerotic rats. The expression of eNOS was reduced, while those of MAPK and NF-κBp38 proteins were upregulated in the aortic tissue homogenate of atherosclerosis group, when compared with the normal group of rats. However, there was significant increase in eNOS activity and marked decreases in the expression of MAPK and NF-κBp38 in the aortic tissue homogenate of AC-treated group, relative to the atherosclerosis group of rats.

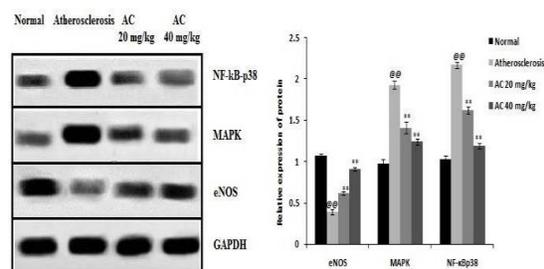


Figure 2: Effect of AC on the protein expressions of NF-κB, eNOS and p38 MAPK in the tissue homogenate of atherosclerotic rats. Data are mean ± SEM (n = 8); @@p < 0.01, vs normal group; *p < 0.05, **p < 0.01, vs atherosclerosis group

Effect of AC on mRNA expressions of ICAM-1, VCAM-1 and MCP-1

Figure 3 shows the effect of AC on the mRNA expressions of ICAM-1, VCAM-1 and MCP-1 in the aortic tissue homogenate of atherosclerotic group. The mRNA expressions of ICAM-1, VCAM-1 and MCP-1 were increased significantly (p < 0.01) in atherosclerosis group, when compared with the normal group of rats. However, AC treatment led to significant and dose-dependent reductions in the mRNA expressions of ICAM-1, VCAM-1 and MCP-1 in the aortic tissue homogenate, when compared with the atherosclerosis group.

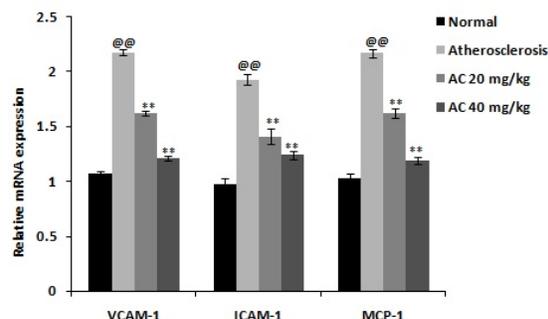


Figure 3: Effect of AC on the mRNA expressions of VCAM-1, ICAM-1 and MCP-1 protein in the tissue homogenate of atherosclerotic rats. Data are mean ± SEM (n = 8); @@p < 0.01, relative to normal group; *p < 0.05, **p < 0.01, relative to atherosclerosis group

Effect of AC on the histopathology of aortic tissues

Figure 4 shows the effect of AC on the histopathology of aortic tissue in atherosclerotic rats, and the ratio of I to I + M. There was normal arrangement of endothelial cells, and the morphology of aortic tissue was normal in the normal control group of rats. However, aortic tissue of atherosclerosis group showed accumulation of foam cells, proliferation of endothelial cells, and disarrangement of these cells. Tunica media thickness of artery was also higher in atherosclerosis group. However, in AC-treated group, the histopathology of aortic tissue

showed normal arrangements in the thin tunica media layer and endothelial cells (Figure 4 A). The ratio of I to I + M was higher in atherosclerosis group than in normal group of rats. Treatment of AC reduced the ratio of I to I + M in a dose-dependent manner, when compared to atherosclerosis group (Figure 4 B).

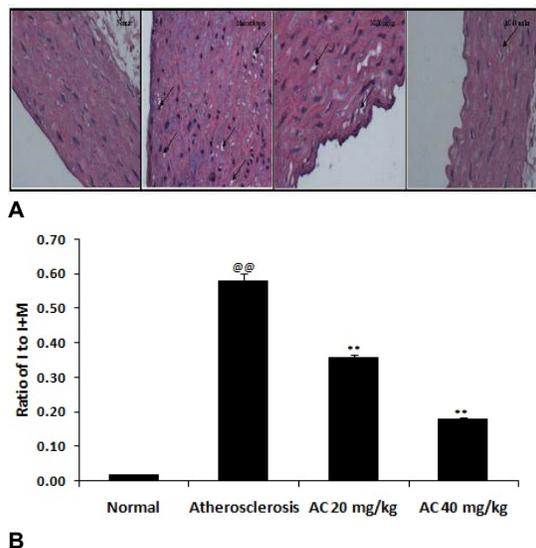


Figure 4: Effect of AC on histopathology of aortic tissue in atherosclerotic rats A: TS of aortic tissue; B: Ratio of I to I + M. Data are mean \pm SEM (n = 8); @@ p < 0.01, compared to normal group; ** p < 0.05, ** p < 0.01, compared to atherosclerosis group

DISCUSSION

Atherosclerosis refers to formation of atheroma on the aortic wall due to increase in serum concentration of lipids [10]. In this study, atherosclerosis was induced by administration of high fat diet, and the effect of AC was determined by estimating serum lipid profiles and mediators of inflammation and parameters of oxidative stress in the aortic tissue. Western blot assay and RT-PCR were performed for the estimation of expression of proteins. Hypercholesterolemia is one of the major factors in the development of atherosclerosis, and the rat model of high fat diet-induced atherosclerotic has been reported in the literatures [11]. The results of this study suggest that treatment with AC reduces the concentration of serum lipids in atherosclerotic rats. In hypercholesterolemic rats, lipid profiles in serum and mediators of inflammation in the aortic tissue are elevated [12]. Several factors contribute to the development of atherosclerosis, including uncontrolled inflammatory response. Studies have shown that inflammatory cytokines activate macrophages and induce the proliferation of

endothelial cells [13]. In addition, it has been reported that the development of atherosclerosis is controlled by some anti-inflammatory drugs. The results obtained in the present study suggest significant reductions in the serum levels of mediators of inflammation in the AC treated group, relative to the atherosclerosis group of rats. Oxidative stress is also responsible for the development of atherosclerosis and endothelial injury [14].

Data from this study suggest that treatment with AC attenuated the altered levels of oxidative stress parameters in the aortic tissue of atherosclerotic rats. Deregulation of eNOS/NO pathway enhanced atherogenesis and inflammation in the aortic tissue, but AC treatment attenuated the altered eNOS/NO signaling pathway in the aortic tissue of atherosclerotic rats [15]. Moreover, the protein expressions of NF- κ B and MAPK were attenuated in the aortic tissue of AC-treated group, relative to the atherosclerosis group of rats.

The expressions of the inflammatory molecules MCP-1, VCAM-1 and ICAM-1 are enhanced in atherogenesis due to endothelial dysfunction [16]. Studies have shown that the expressions of VCAM-1 and ICAM-1 are upregulated due to impairment of NO [17]. Moreover, in endothelial cells, expression of MCP-1 is enhanced due to inhibition of NO synthesis [18]. The results of this study have revealed that treatment with AC downregulated the mRNA expressions of MCP-1, VCAM-1 and ICAM-1 in the aortic tissue homogenate of atherosclerotic rats.

CONCLUSION

The findings in this investigation suggest that AC mitigates atheroma formation in the artery of atherosclerotic rats. Treatment with AC has beneficial effects on serum lipid profiles and inflammatory cytokines, as well as oxidative stress parameters in the aortic tissue of atherosclerotic rats. Thus, AC prevents atherosclerosis in rats through regulation of the NF κ B/eNOS/NO signaling pathway, indicating its potential for clinical application in the management of atherosclerosis.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xianghua Xu conducted the experimental work and literature review related to presented work. Honggang Pang, Hao Qin and Qian Yin performed the molecular assay and histopathological analysis. Qiang Ma, Junbo Zhang and Bo Zhang performed the statistical analysis and Yan Meng supervised the work.

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