Original Research

Triacetyl-3-hydroxyphenyladenosine, a derivative of cordycepin, attenuates atherosclerosis in apolipoprotein E-knockout mice

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Abstract

The cholesterol-modulating, immune-regulating and anti-inflammatory properties of cordycepin are well documented. Here we examined the effects of triacetyl-3-hydroxyphenyladenosine (THPA), a derivative of cordycepin, on the development of atherosclerosis (AS) in apolipoprotein E-knockout (apoE$^{-/-}$) mice. The atherosclerotic lesion formation displayed by the oil red O staining-positive area was reduced significantly in either the aortic root section or the whole aorta en face in THPA-administrated apoE$^{-/-}$ mice. Plasma analysis by enzymatic method or enzyme-linked immunosorbent assay (ELISA) showed that high-density lipoprotein-cholesterol (HDL-C) was decreased, whereas apolipoprotein A-I (apoA-I) levels were markedly increased by THPA. In addition, ELISA and spectrophotometric measurement showed that plasma levels of tumor necrosis factor-α, interleukin-1 and malondialdehyde were decreased in mice treated with THPA. Realtime polymerase chain reaction detection disclosed that the expression of several transporters involved in reverse cholesterol transport was induced by THPA, and the expression of hepatic ABCA1 and apoA-I, which play roles in the maturation of HDL-C, was also elevated in the THPA-treated groups. Moreover, THPA enhanced the expression of endothelial nitric oxide synthase (NOS), and reduced the expression of inducible NOS and lectin-like oxidized LDL receptor-1 in the aorta, suggesting that THPA can exert endothelial protection effects. In addition, the expression or activation of several proinflammatory factors in the aorta was suppressed by THPA. In conclusion, our results reveal the inhibitory effects of THPA on AS in apoE$^{-/-}$ mice.

Keywords: atherosclerosis, triacetyl-3-hydroxyphenyladenosine, reverse cholesterol transport related genes, vascular wall inflammation, apolipoprotein A-I

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Introduction

Atherosclerosis (AS) is a condition of multifactorial chronic vascular inflammatory lesions that leads to the onset of cardiovascular disease (CVD).[1] Inflammatory processes within the vessel wall mediate the initiation and progression of AS. Multiple inflammatory factors and genes are involved in all stages of AS processes. Monocyte invasion into lesion-prone areas in the arterial wall, and differentiation of monocytes into resident macrophages, contribute to atherosclerotic plaque development. Current strategies to reduce AS are focusing on the anti-inflammatory therapy and primarily target reducing the influx of cholesterol into the arterial wall by lowering plasma low-density lipoprotein-cholesterol (LDL-C) concentrations. Aggressive lowering of plasma LDL levels reduces mortality from coronary heart disease,[2] but protection is not complete, and in a significant proportion of patients, plasma LDL concentrations cannot be lowered to a level that would be predicted to halt the progression of disease. As a consequence, there is a significant interest in strategies aimed at enhancing cholesterol efflux from the arterial wall and promoting its transport to the liver for excretion in a process called reverse cholesterol transport (RCT).

Cordycepin, also known as 3′-deoxyadenosine, is one of the main active constituents of the traditional herbal medicine *Cordyceps militaris*, with a broad spectrum of biological activities, including immunoregulational, anti-inflammatory,[3] anti-diabetic[4] and also the inhibition of platelet aggregation.[5] Cordycepin has also been shown to have cholesterol-reducing[6] and anti-AS properties.[7] Triacetyl-3-hydroxyphenyladenosine (THPA) compound (patent number WO/2010/040286), a type of cordycepin-derivative, was synthesized by the Chinese Academy of Medical Sciences (Beijing, China), and declared to have

hypolipidemic activity, but its effects on atherogenesis have not been elucidated. In the present study, we hypothesized that THPA might inhibit the progression of AS, bearing in mind the beneficial effects of cordycepin and its derivatives on vascular biology. Therefore, we investigated the atheroprotective effects of THPA compound and the underlying mechanisms in apolipoprotein E-knockout ($apoE^{-/-}$) mice, a spontaneously developed AS model.

Materials and methods

Animals and experimental design

Forty-one male $apoE^{-/-}$ mice were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences. All experiments were approved by the Laboratory Animals' Ethical Committee of Taishan Medical University and followed national guidelines for the care and use of animals. At five weeks of age, the 41 $apoE^{-/-}$ mice were randomly divided into four groups, the model group ($n = 11$, vehicle-treated group), and three THPA (2.5, 5.0 and 10 mg/kg/d)-treated groups ($n = 10$ each group). All groups were fed an atherogenic high-fat diet (15.8% fat and 1.25% cholesterol) supplemented with vehicle or THPA for six weeks. Vehicle or THPA was administered intragastrically once daily. Caboxymethyl cellulose sodium salt solution (0.5%) was used as vehicle for dissolving THPA.

Synthesis of THPA

THPA compounds were synthesized as described previously[8] and provided by Professor Haibo Zhu (Chinese Academy of Medical Sciences).

Plasma analyses

After six weeks of treatment, blood was collected from the retro-orbital sinus of the mice without dietary exposure for 12 h. Concentrations of plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were determined by enzymatic methods (BioSino, Beijing, China). Non-HDL-C was calculated as TC minus HDL-C. Plasma concentrations of apolipoprotein A-I (apoA-I) were determined by enzyme-linked immunosorbent assay (ELISA) kit (BlueGene, Shanghai, China). Endogenous lecithin-cholesterol acyltransferase (LCAT) activity was measured as the utilization rate of free cholesterol (FC) in native plasma according to the method by Ly *et al.*[9] The FC content was measured colorimetrically by using a kit (BioVision, Mountain View, CA, USA) in pentuplicate by a microplate reader (Tecan, Zurich, Switzerland) at zero time and after one hour at 37°C. LCAT activity was expressed as nanomoles FC consumed per hour per milliliter plasma. Plasma phospholipid transfer protein (PLTP) activity was measured as previously described.[10] Plasma levels of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and high-sensitivity C-reactive protein (hsCRP) were measured by ELISA kit (BlueGene). Plasma malondialdehyde (MDA) content was determined by a spectrophotometric measurement of thiobarbituric acid-reactive substances according to the manufacturer's instructions (Nanjing Jiancheng Biochemistry, Nanjing, China).

Lesion analysis

The proximal aorta attached to the heart was used to prepare cross-sections. Cryosections (8 μm) were cut from the site where the aorta valve cups appear at the aorta root, collected and stained with oil red O and brilliant green. The volume of stained lipids (μm^2) was calculated from five sections per animal. For determination of the atherosclerotic plaque area in the whole aorta, the whole aorta were dissected and stained en face with oil red O. Quantitative analysis of the plaque area was performed by two blinded observers using Image-Pro Plus software 4.5 (Media Cybernetics, Rockville, MD, USA).

Western blots

Tissues or cells were harvested and protein extracts prepared as previously described.[11] They were then subjected to Western blot analyses using anti-lectin-like oxidized LDL receptor (LOX-1) antibody (Abcam, Cambridge, MA, USA), and anti-ATP-binding cassette transporter A1 (ABCA1), ABCG1, scavenger receptor BI (SR-BI), apoA-I and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The proteins were visualized and quantified using a chemiluminescence method (Thermo Scientific Pierce, Rockford, IL, USA) and Quantity One (Bio-Rad, Hercules, CA, USA) software program.

Immunohistochemistry analysis

Serial aortic root cryosections (8 μm) were stained with specific antibodies against vascular cell adhesion molecule-1 (VCAM-1) (Santa Cruz Biotechnology), tissue factor (TF) (Santa Cruz Biotechnology), nuclear factor-κB (NF-κB) p65 (Abcam), LOX-1 (Abcam), endothelial nitric oxide synthase (eNOS) (Santa Cruz Biotechnology), inducible NOS (iNOS) (Santa Cruz Biotechnology) and TNF-α (Abcam). The sections were developed with 3,3′-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) and counterstained with Mayer's hematoxylin. Images were captured using microscope (Olympus, Tokyo, Japan). Color threshold and planimetry were used for quantification. All quantifications were determined by calculating the percentage of the antigen-positive area to the total cross-sectional vessel wall area by using Image-Pro Plus software.

Isolation of peritoneal macrophages

Three days after intraperitoneal thioglycollate injection, peritoneal macrophages were harvested from six-week THPA- or vehicle-treated $apoE^{-/-}$ mice. Following collection, peritoneal macrophages were immediately frozen at −80°C until RNA or protein extraction.

Quantitative realtime polymerase chain reaction

Total cellular RNA was isolated by TRIZOL Reagent (Invitrogen, Grand Island, NY, USA). cDNA synthesis was

performed using MuLV Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA). Realtime polymerase chain reaction (PCR) was performed using a SYBR-green PCR master mix kit (TianGen Biotech, Beijing, China). The primers used for realtime PCR are listed in Table 1. The data were analyzed by using Rotor-gene Q software version 1.7 (Qiagen, Valencia, CA, USA). Relative mRNA levels were calculated by the method of 2^{-DDCt}.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance test with GraphPad Prism program version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as means $\pm$ SD. P values less than 0.05 were considered significant.

Results

THPA decreases atherosclerotic lesion formation

All dosages of THPA (2.5, 5.0 and 10 mg/kg) significantly inhibited the formation of atherosclerotic lesions in cross-sections of the aortic valve area by 40%, 33% and 35%, respectively, compared with the model group (Figure 1a and c). En face oil red O staining of the whole aorta also revealed a significant 31%, 33% and 35% reduction of atherosclerotic plaque formation in animals treated with 2.5, 5.0 and 10 mg/kg of THPA, respectively (Figure 1b and d).

Effect of THPA on plasma lipoprotein profiles, plasma PLTP and LCAT activities

As shown in Table 2, plasma TC, TG and non-HDL-C levels after six weeks of treatment were not significantly affected by THPA treatment, although levels tended to be lower after treatment. However, plasma apoA-I (the major structural component of HDL) levels in mice fed 2.5, 5.0 and 10 mg/kg of THPA were significantly higher than that in the model group. Interestingly, plasma levels of HDL-C were decreased after THPA treatment, suggesting that the rate of HDL-C catabolism might be increased by THPA. In addition, different dosages of THPA (2.5, 5.0 and 10 mg/kg) significantly decreased plasma PLTP activities ($P < 0.05$) and increased plasma LCAT activities ($P < 0.01$).

Effect of THPA on the expression of hepatic genes involved in cholesterol metabolism

As shown in Figure 2a, THPA treatment increased the mRNA levels of hepatic apoA-I and ABCA1 up to 1.53 ± 0.2 fold ($P < 0.05$) and 1.93 ± 0.26 fold ($P < 0.01$), respectively. The mRNA levels of ABCG5 and ABCB11 were also elevated by THPA treatment. In addition, the mRNA levels of ABCG1, apoA-II, SR-BI, LCAT and ABCG8 were not affected by THPA. This difference in hepatic LCAT mRNA levels was not reflected in plasma LCAT activity, suggesting that post-transcriptional regulation plays an important role in LCAT function. Furthermore, as shown in Figure 2b and c, the protein levels of ABCA1 and apoA-I were elevated and the levels of ABCG1 and SR-BI were not affected by THPA treatment. The fold induction of ABCA1 and apoA-I protein levels was higher than that of the mRNA levels, suggesting that post-transcriptional regulation partially plays a role in ABCA1 and apoA-I expression.

Table 1 Primers used for realtime polymerase chain reaction analysis

Gene	Primer	Sequence (5′-3′)
ABCA1	Sense	CGTTTCCGGGAAGTGTCCTA
	Antisense	GCTAGAGATGACAAGGAGGATGGA
ABCG1	Sense	GGGAAGTTGATAAAGGATGT
	Antisense	GATTCGGGCTATGTATGG
SR-BI	Sense	ATCTGGTGGACAAATGGAA
	Antisense	GAAGCGATACGTGGGAAT
ApoA-I	Sense	GGCACGTATGGCAGCAAGAT
	Antisense	CCCAGAAGTCCCGAGTCAAT
ApoA-II	Sense	CCAAGGCATACTTTGAGAAGACAC
	Antisense	GGAGAAAACAGGCAGAAGGTAGG
LCAT	Sense	CCCACCAGCAGGATGAATACTAC
	Antisense	AGGCTATGCCCAATGAGGAA
ABCG5	Sense	AGCGTCAGCAACCGTGTC
	Antisense	AGCAGCGTGGTCTTCCCT
ABCG8	Sense	TTAAGCCACTCCCAATACA
	Antisense	GTTGCTCCAAGAATAAATGA
ABCB11	Sense	CAAATAAGGTTGTGGGTAA
	Antisense	AGGACTGACAGCGAGAAT
LOX-1	Sense	CAAAGTCTCCCAACCAAC
	Antisense	GAGGCATACTGAGGTAGAGG
GAPDH	Sense	TGACGTGCCGCCTGGAGAAA
	Antisense	AGTGTAGCCCAAGATGCCCTTCAG

Effect of THPA on the expression of transporter genes involved in cholesterol efflux in peritoneal macrophages

The effect of THPA on the expression of macrophage ABCA1, ABCG1 and SR-BI, which play important roles in cholesterol efflux from peripheral tissues, was determined. Western blot analysis showed that SR-BI expression was significantly increased and the expression of ABCA1 and ABCG1 was not affected by THPA treatment (Figure 3a and b). Also, realtime PCR analysis displayed significantly increased SR-BI mRNA expression levels in the THPA-fed groups and analysis of ABCA1 and ABCG1 mRNA showed no difference among the four groups (Figure 3c).

THPA reduces inflammatory processes in the arterial wall

Quantitative immunohistochemistry imaging revealed that the positive area of VCAM-1, TNF-α and TF within the lesion area was significantly decreased (68% reduction of VCAM-1, 70% reduction of TNF-α and 66% reduction of TF) in mice treated with THPA compared with the model group (Figure 4a–c, f–h). Besides, the expression of iNOS, a macrophage marker, was also down-regulated by 50%, suggesting an inhibition of macrophage activation by THPA (Figure 4d and i). As shown in

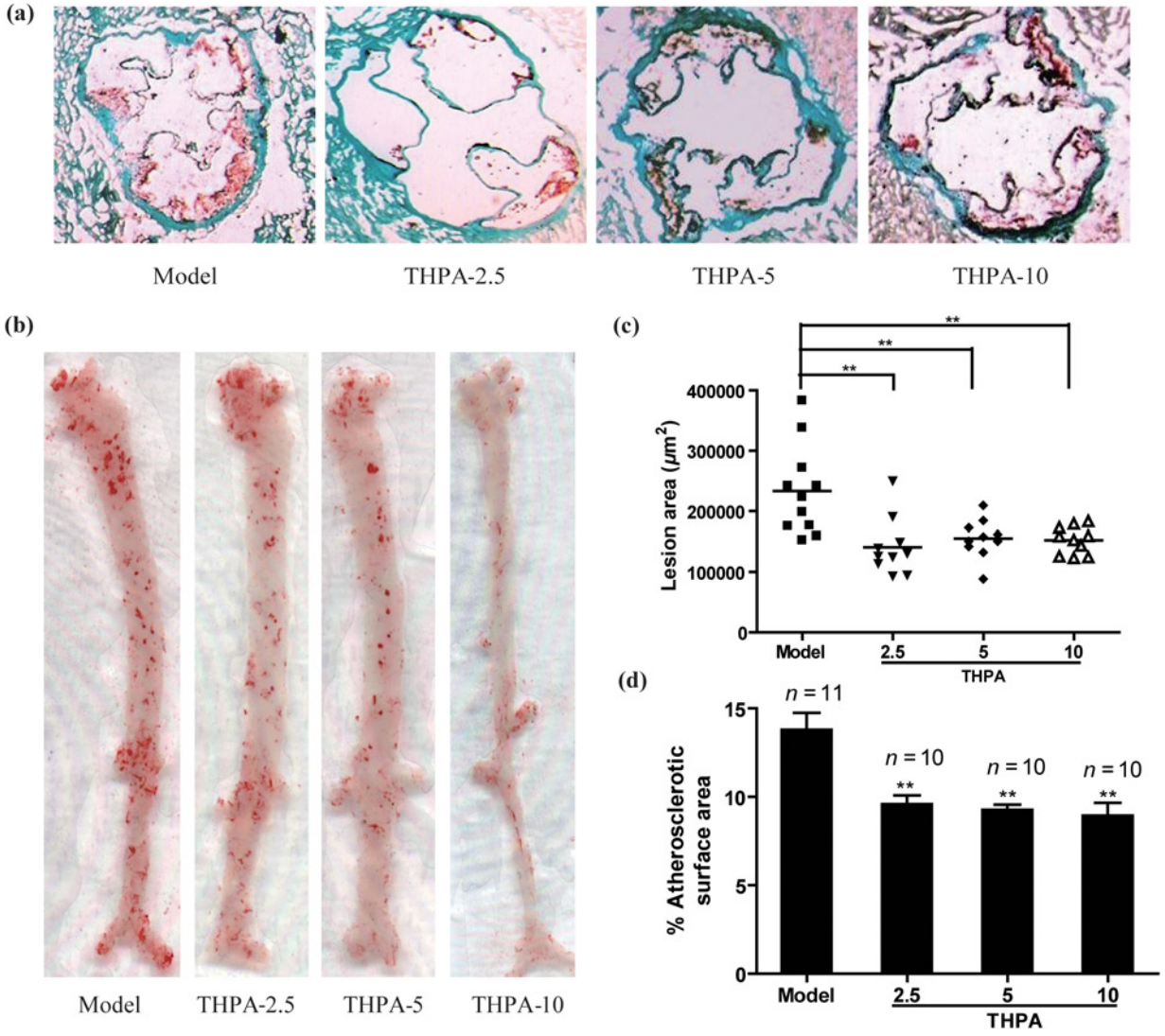

Figure 1 THPA inhibits atherosclerotic lesion formation. (a) Representative of oil red O-stained aortic sections (×10 magnification). (b) Representative of en-face oil red O staining of the whole aorta. (c) Quantitation of lesion areas in oil red O-stained aortic sections by Image-Pro Plus software. (d) Quantification of atherosclerotic lesions in the en face of the whole aorta. ** $P < 0.01$ versus model group. THPA, triacetyl-3-hydroxyphenyladenosine. (A color version of this figure is available in the online journal)

Figure 4e and j, the number of cells showing active (nucleus-associated) p65-NF-κB immunoreactivity in the THPA-treated groups was reduced by 59%. These results indicate that THPA reduces the number of active p65-NF-κB-containing cells, providing a molecular explanation for the reduced inflammation in the THPA-treated groups.

Table 2 Effect of THPA on plasma lipoprotein lipids, apoA-I content, PLTP and LCAT activity in apoE$^{-/-}$ mice

	Model	THPA 2.5	THPA 5	THPA 10
Animal number (*n*)	11	10	10	10
TC (mmol/L)	20.40 ± 5.27	16.10 ± 4.00	18.69 ± 4.15	18.10 ± 2.76
TG (mmol/L)	2.14 ± 0.58	1.65 ± 0.58	1.80 ± 0.34	2.14 ± 0.43
HDL-C (mmol/L)	5.73 ± 1.53	4.20 ± 0.65**	4.34 ± 0.74**	3.99 ± 0.71**
Non-HDL (mmol/L)	14.67 ± 4.38	11.20 ± 4.00	13.60 ± 3.47	14.11 ± 2.66
ApoA-I (mg/dL)	4.24 ± 1.02	15.13 ± 4.71**	13.80 ± 3.05**	12.73 ± 2.29**
PLTP activity (pmol/μL/hour)	211.8 ± 36.5	179.7 ± 36.8*	181.2 ± 29.4*	179.9 ± 30.8*
LCAT activity (nmol/mL/hour)	294.4 ± 85.32	1415.9 ± 86.56**	1383.1 ± 82.48**	1532.08 ± 158.86**
Body weight (g)	21.8 ± 1.2	21.3 ± 1.4	20.9 ± 1.8	21.1 ± 1.6

TC, total cholesterol; TG, triglycerides; THPA, triacetyl-3-hydroxyphenyladenosine; HDL-C, high-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; PLTP, plasma phospholipid transfer protein; LCAT, lecithin-cholesterol acyltransferase
Values are the means ± SD
*Compared with model, $P < 0.05$
**Compared with model, $P < 0.01$

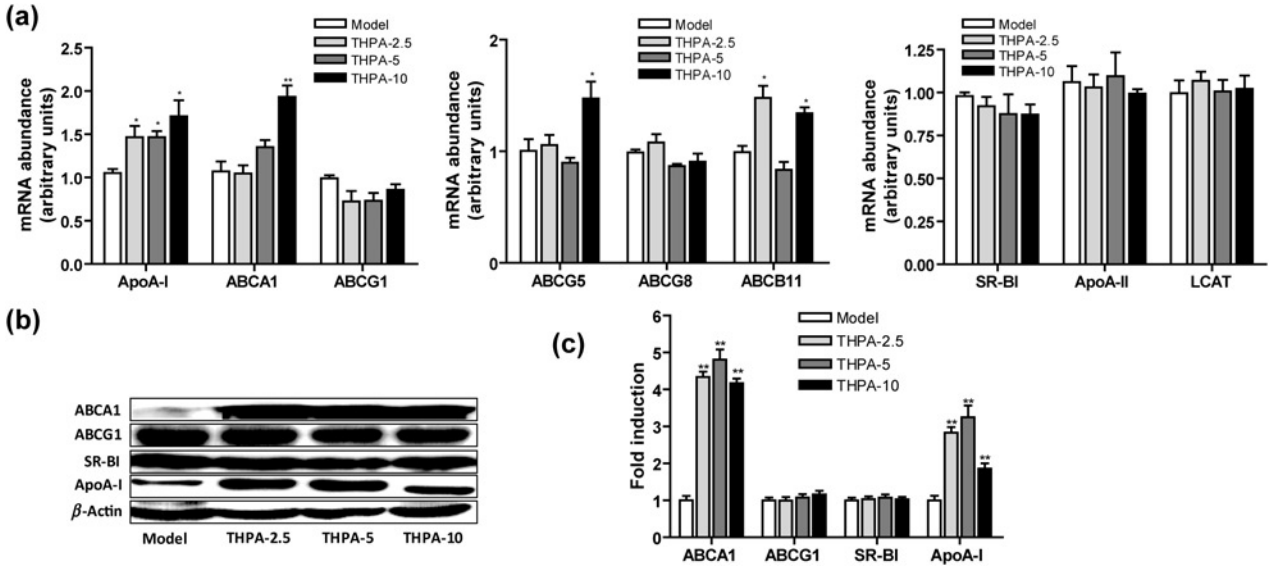

Figure 2 Effect of THPA on the expression of hepatic genes involved in cholesterol metabolism. (a) Effect of THPA on the mRNA expression of hepatic genes by realtime PCR. Expression levels of mRNA are indicated as fold differences compared with model mice. (b) Effect of THPA on the protein expression of hepatic genes by Western blots. (c) Densitometric quantitation of Western blot data ($n = 5$) by Quantity One software. $^{*}P < 0.05$, $^{**}P < 0.01$ versus model group. THPA, triacetyl-3-hydroxyphenyladenosine; PCR, polymerase chain reaction

Effect of THPA on plasma levels of proinflammatory and antioxidant markers

As shown in Figure 5a and b, plasma levels of TNF-α and IL-1, the proinflammatory cytokines, were both decreased in THPA-treated mice, suggesting that THPA might affect the inflammatory process in plasma. However, the plasma level of hsCRP was not affected by THPA treatment (Figure 5c). In addition, plasma levels of MDA, one of the most frequently used indicators of lipid peroxidation, were decreased (Figure 5d) in mice treated with different dosages of THPA, suggesting that THPA treatment impairs lipid oxidation and peroxidation in plasma.

Effect of THPA on the expression of eNOS and LOX-1 in the arterial wall

Immunohistochemistry data showed a 1.9-fold induction of eNOS gene expression in the aortic wall in THPA-treated animals (Figure 6). Western blots (Figure 7a) and quantitative realtime PCR (Figure 7b) revealed that the expression of LOX-1 in the aortas was significantly decreased by 75% in protein levels and 77% in mRNA levels in mice treated with THPA compared with the model group. This was confirmed by immunohistochemistry imaging showing significantly lower expression of LOX-1 within the lesion area of THPA-treated animals (Figure 7c).

Discussion

In the present study, we evaluated the antiatherosclerotic effect of the synthesized drug THPA, a compound derived from cordycepin, in an AS-prone murine model. THPA-treated mice allowed us, for the first time, to quantify and to comprehensively characterize the antiatherosclerotic effects of THPA *in vivo*. THPA has been declared to have hypolipidemic activity in rats and hamsters (patent number WO/2010/040286); however, the expected cholesterol-lowering effect of THPA was not observed in our apoE$^{-/-}$ mice, which is a spontaneously developed AS model. This phenomenon might be explained by the severe dyslipidemic profile of apoE$^{-/-}$ mice whose lipid profile is very-low-density lipoprotein-dominant. In this study, we found that the THPA compound significantly inhibited the aortic plaque load and cross-sectional lesion area. The effect of THPA was paralleled by the down-regulation of plasma PLTP activities, the increased levels of factors important for RCT (macrophage SR-BI expression, plasma apoA-I and LCAT activity, hepatic ABCG5 and ABCB11 expression), the improved endothelial function (eNOS and LOX-1) and the strong reduction of aortic inflammation (VCAM-1, TF, p65-NF-κB, iNOS and TNF-α).

It has been reported that cordycepin, a major bioactive component of traditional fungal drug *Cordyceps militaris*, might be a potent, promising anti-AS agent,[7] but a high production cost and fast metabolism has limited its further development and application. THPA is a chemically synthesized derivative of cordycepin which has the advantages of significant hypolipidemic activity.[6] The principal findings of our study support the notion that THPA can reduce AS beyond the effect attributable to its total plasma cholesterol-lowering effect *per se*, and we also provide a molecular explanation for these additional beneficial effects.

Plasma concentrations of HDL-C and apoA-I have strong inverse correlations with the risk of atherosclerotic CVD, independently of non-HDL-C levels.[12] ApoA-I is the major protein component of HDL-C in plasma. HDL-C and apoA-I exert anti-inflammatory, antioxidant, antithrombotic, promoting RCT and vasodilating properties that could all potentially be involved in their antiatherogenic effects.[13] The metabolism of HDL is a complex process, in which the individual lipid and apolipoprotein components

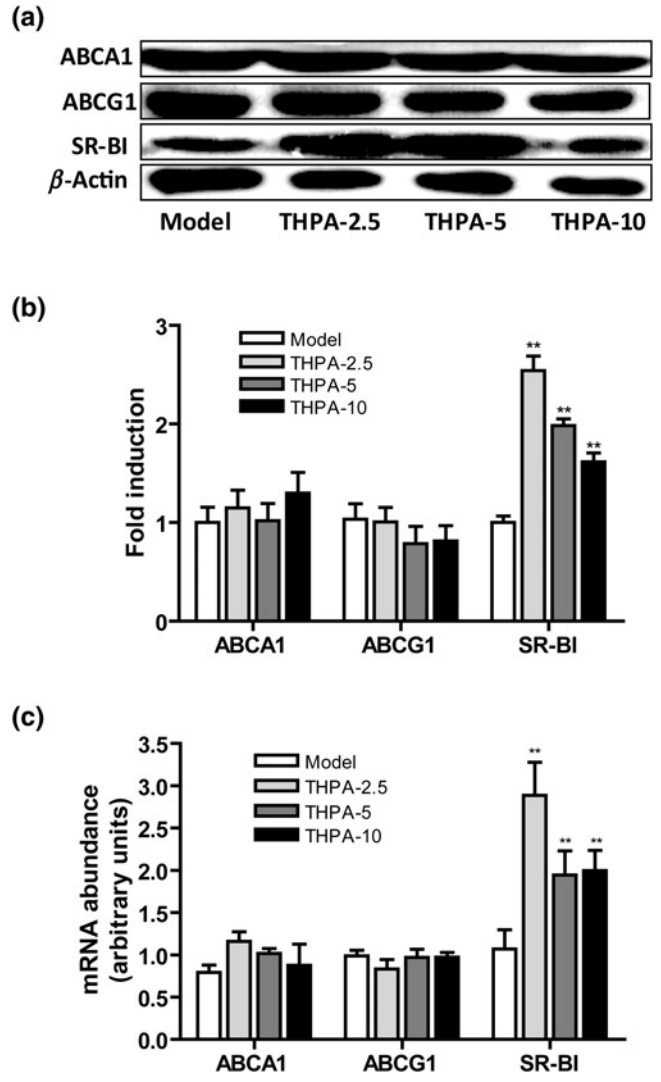

Figure 3 Effect of THPA on the expression of transporter genes involved in cholesterol efflux in peritoneal macrophages. (a) Effect of THPA on the protein expression of macrophage transporter genes by Western blots. (b) Densitometric quantitation of Western blot data ($n = 5$) by Quantity One software. (c) Effect of THPA on the mRNA expression of macrophage transporter genes by realtime PCR. Expression levels of mRNA are indicated as fold differences compared with model mice. $^{**}P < 0.01$ versus model group. THPA, triacetyl-3-hydroxyphenyladenosine; PCR, polymerase chain reaction

of HDL are mostly assembled after secretion, are frequently exchanged with or transferred to other lipoproteins, are actively remodeled within the plasma compartment, and are cleared in the kidney or in the liver, at least in part, independent from one another.[14] Metabolic turnover studies suggest that plasma levels of HDL-C and apoA-I are determined by the rate of synthesis versus the rate of catabolism,[14] and the variation in plasma HDL-C and apoA-I concentrations in the general population, is primarily a function of variation in clearance or production rates.[14] Here, we showed, for the first time, that THPA treatment increased plasma levels of apoA-I whereas it decreased plasma HDL-C levels. These data gave us a clue that THPA might enhance the production of apoA-I and also increase the rate of HDL-C catabolism. We all know that small HDL particles, especially apoA-I, could promote cholesterol efflux from tissues to the liver for excretion; therefore, the function of the increased apoA-I in our study needs to be explored in the future.

It is well known that plasma PLTP mediates both net transfer and exchange of phospholipids between different lipoproteins. Our previous data have shown that PLTP-deficient mice have demonstrated increased antioxidation potential as well as a decrease in apolipoprotein B (apoB) secretion and atherosclerotic lesions.[15,16] In this study, we found that plasma PLTP activity was decreased by THPA treatment; therefore, it is necessary to test whether the decreased plasma PLTP activity in our study is closely associated with low plaque burden by using PLTP-transgenic mice in the future. In addition, PLTP has been reported to modify HDL by the mechanism which involves particle fusion,[17,18] resulting in the remodeling of HDL primarily into larger particles with loss of apoA-I as well as into smaller particles that are rich in apoA-I.[18] We know that the larger HDL particles are not easy to be catabolized. Correspondingly, several reports have shown that PLTP activity was positively related to HDL-C,[19] and in patients with low HDL and CVD, PLTP activity was positively correlated with the concentration of HDL particles.[20] Therefore, in our study, it is possible that the descended plasma HDL-C level might be attributable, at least in part, to the down-regulated PLTP acitivities in THPA-treated animals. Next, apoA-I is mainly synthesized by the liver and hepatic ABCA1 exerts an antiatherogenic effect via its contribution to HDL-C formation by promoting lipid efflux to apoA-I from hepatocytes.[21] Our results showed that the expressions of hepatic apoA-I and ABCA1 were increased by THPA treatment *in vivo*; these data further support our prediction that THPA could enhance the synthesis of apoA-I and in the meanwhile increase the rate of HDL-C catabolism, at least in part, by the PLTP-mediated pathway.

RCT is the pathway by which peripheral cell cholesterol can be returned to the liver for catabolism.[22] Recently, several key molecules have been identified to play pivotal roles on RCT.[23–25] Macrophage ABCA1 facilitates cholesterol efflux from cells to lipid-poor apoA-I, but not HDL,[23,24] whereas another two macrophage transporters, ABCG1 and SR-BI, are involved in the cholesterol efflux from macrophages mediated by HDL, but not apoA-I.[25,26] In our study, we showed, for the first time, that THPA compound induced macrophage SR-BI expression, which might lead to an increased HDL-mediated cholesterol efflux from macrophages. Furthermore, LCAT which bound to HDL in plasma and plays a role in RCT, is an enzyme that converts free cholesterol into cholesteryl ester and is critical for the maturation and maintenance of normal HDL metabolism in humans[27] and mice.[28] At present, the molecular mechanism(s) that regulates plasma LCAT concentrations is not well understood. LCAT activity depends in part on the amount of the enzyme in plasma, and in part on the substrate and co-factors available to the enzyme,[29] such as apoA-I, an activator of LCAT, and apoA-II, an inhibitor of LCAT activity. Our results showed that THPA treatment did not influence the mRNA expression of hepatic LCAT and apoA-II, but significantly increased plasma LCAT

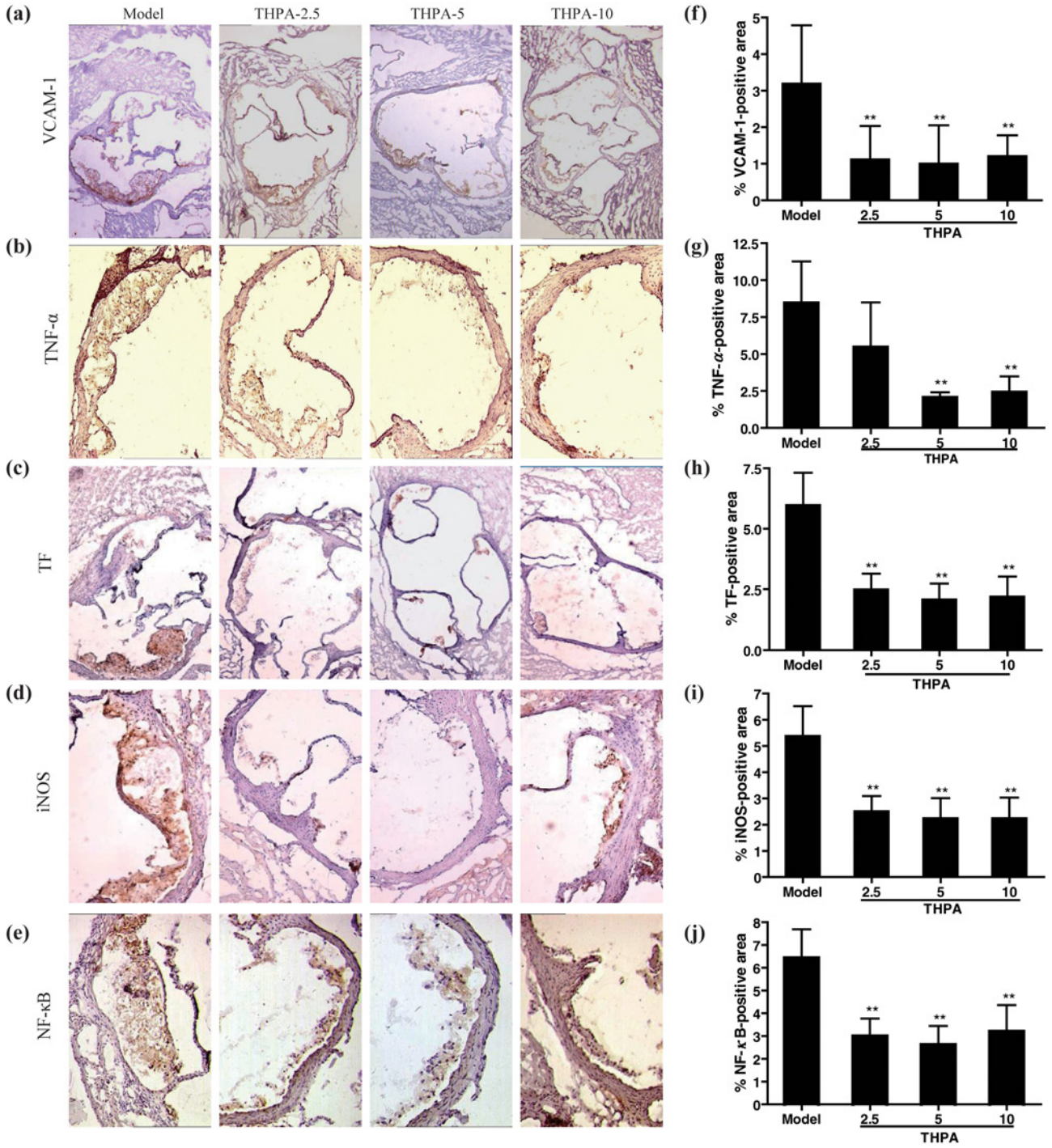

Figure 4 THPA reduces inflammatory processes in the arterial wall. (a–e) Representative of immunostained aortic sections using specific antibodies against VCAM (×10 magnification; blue = nuclei, brown = VCAM), TNF-α (×20 magnification; blue = nuclei, brown = TNF-α), TF (×10 magnification; blue = nuclei, brown = TF), iNOS (×20 magnification; blue = nuclei, brown = iNOS) and NF-κB (×20 magnification; blue = nuclei, brown = NF-κB), respectively. (f–j) Densitometric quantification of VCAM-, TNF-α-, TF-, iNOS- and NF-κB-positive cells per field of view. $^{**}P < 0.01$ versus model group. THPA, triacetyl-3-hydroxyphenyladenosine; VCAM, vascular cell adhesion molecule; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase; TF, tissue factor; NF-κB, nuclear factor-κB. (A color version of this figure is available in the online journal)

activities, hepatic apoA-I and plasma apoA-I concentrations. The discrepancy in hepatic LCAT expression and plasma LCAT activity might be attributable to a post-transcriptional regulation of LCAT or the upregulation of apoA-I, the activator of LCAT, by THPA.

Besides macrophage transporters and plasma LCAT, several hepatic transporters are also involved in the process of RCT. The direct selective uptake of HDL-C by the liver is mediated by SR-BI. However, in our study, the expression of hepatic SR-BI was not altered in THPA-treated mice. After uptake of HDL-associated cholesterol by SR-BI, secretion of cholesterol, bile acids and phospholipids into the bile is regulated by the respective transporters ABCG5, ABCG8 and ABCB11. Here, we

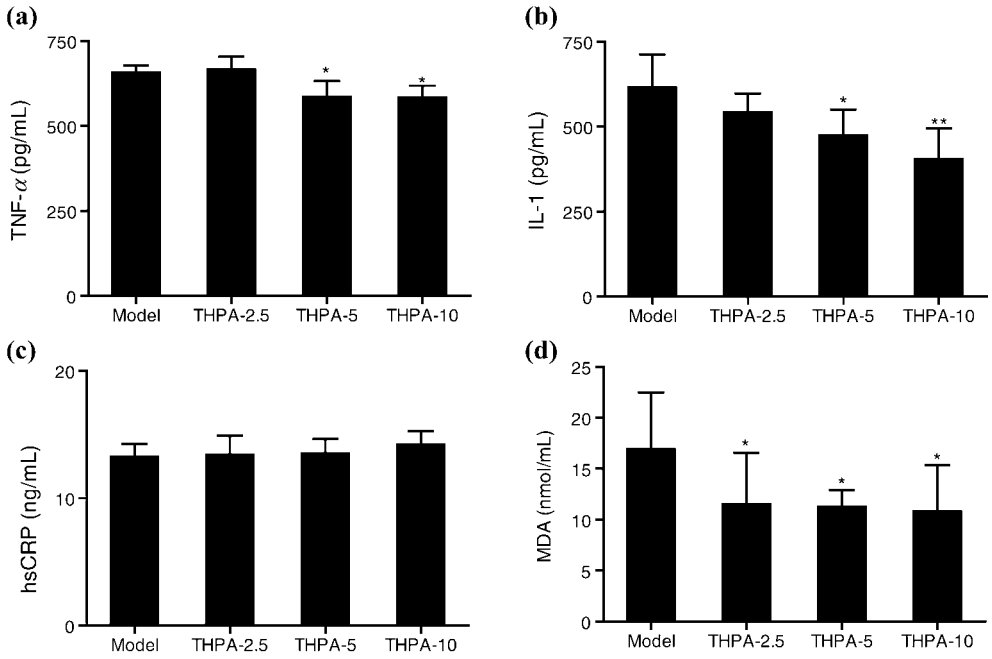

Figure 5 THPA reduces plasma levels of TNF-α, IL-1 and MDA. Effect of THPA on plasma levels of TNF-α (a), IL-1 (b) and hsCRP (c) by ELISA. (d) Effect of THPA on plasma concentrations of MDA content by spectrophotometric measurement. $^{*}P < 0.05$, $^{**}P < 0.01$ versus model group. THPA, triacetyl-3-hydroxyphenyladenosine; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; MDA, malondialdehyde; hsCRP, high-sensitivity C-reactive protein; ELISA, enzyme-linked immunosorbent assay

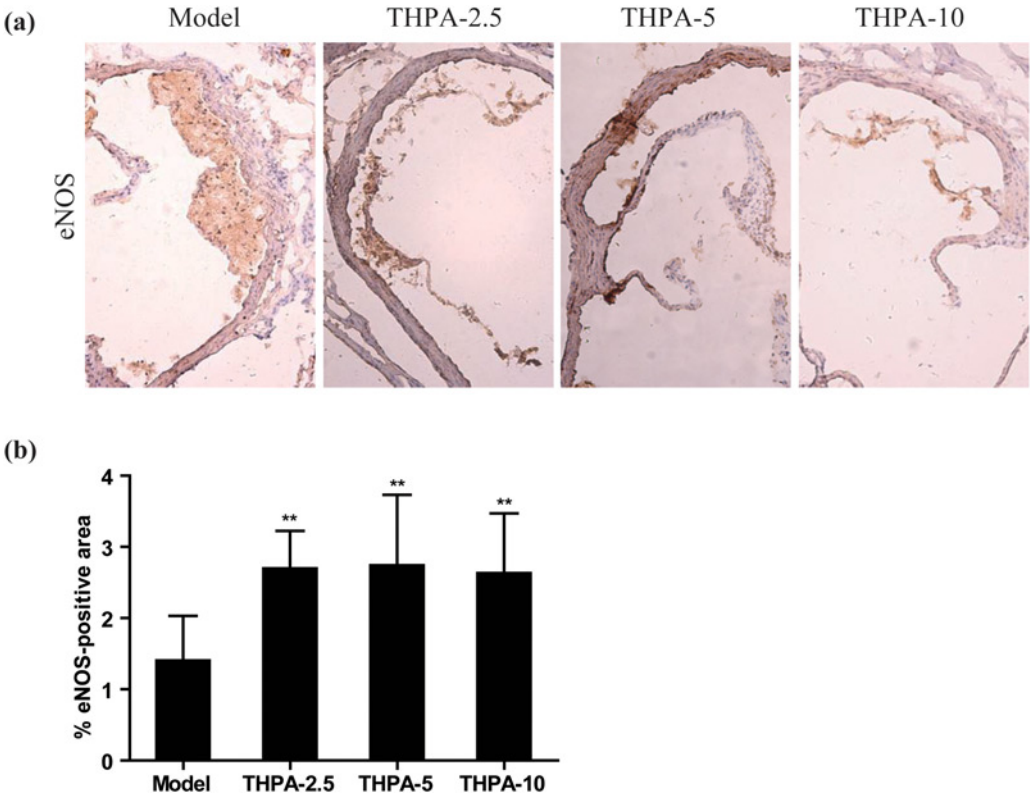

Figure 6 THPA induces eNOS expression in the arterial wall. (a) Representative of immunostained aortic sections using specific antibodies against eNOS ($\times$20 magnification; blue = nuclei, brown = eNOS). (b) Densitometric quantification of eNOS-positive cells per field of view. $^{**}P < 0.01$ versus model group. THPA, triacetyl-3-hydroxyphenyladenosine; eNOS, endothelial nitric oxide synthase. (A color version of this figure is available in the online journal)

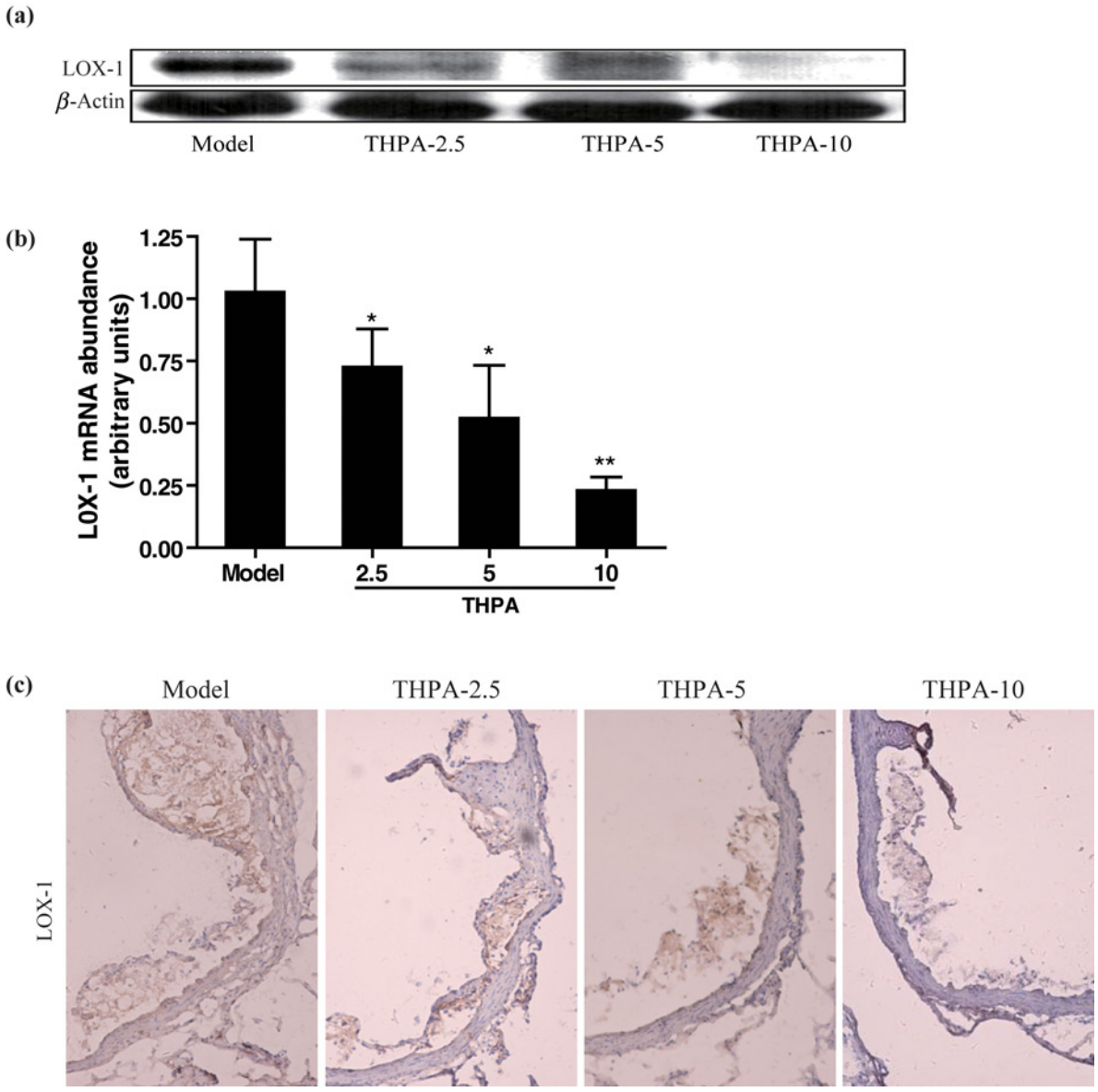

Figure 7 THPA decreases LOX-1 expression in the arterial wall. (a) Western blot analysis of LOX-1 protein expression in the thoracic aorta. (b) Realtime PCR analysis of LOX-1 mRNA expression in the thoracic aorta. Expression levels of mRNA are indicated as fold differences compared with the model mice. (c) Representative of immunostained aortic sections using specific antibodies against LOX-1 (×20 magnification; blue = nuclei, brown = LOX-1). $^{*}P < 0.05$, $^{**}P < 0.01$ versus model group. THPA, triacetyl-3-hydroxyphenyladenosine; LOX-1, anti-lectin-like oxidized LDL receptor; PCR, polymerase chain reaction. (A color version of this figure is available in the online journal)

showed that the mRNA expression of ABCG5 and ABCB11 was up-regulated by THPA treatment. These findings suggest that THPA compound may exert antiatherosclerotic effects by stimulating the expression of transporters involved in the process of RCT, including macrophage SR-BI, hepatic ABCG5 and ABCB11.

Although AS was formerly regarded as a lipid-accumulating disease, it is now widely accepted that inflammatory processes play a critical role in all phases of AS. We found that plasma levels of TNF-α and IL-1, the proinflammatory cytokines, were both decreased in THPA-treated mice, suggesting that THPA might affect the inflammatory process in plasma. Besides, our data provide a possibility that THPA exerts direct anti-inflammatory effects in the aorta by the following evidence: the positive areas of VCAM-1, TNF-α, iNOS (a marker of macrophage activation), TF (a main prothrombotic stimulus found in the atherosclerotic lesion's lipid core) and NF-κB were 68%, 70%, 50%, 66% and 59% smaller, respectively, in the aortic wall of THPA-treated groups. Taken together, the molecular mechanism underlying the anti-inflammatory vascular effects may well be the capacity of THPA to suppress activation of NF-κB (Figure 4), a transcriptional master regulator that controls the expression of adhesion molecules,[30] TF[31] and iNOS.[32] In addition, in the present study, we found increased eNOS and decreased LOX-1 protein levels in the aorta of THPA-fed mice. This suggests that THPA may be able to improve endothelial function. Besides, the down-regulated plasma MDA levels in THPA-treated animals suggest that THPA displayed

antioxidant effects *in vivo*, which potentially contributes to the observed antiatherosclerotic actions of THPA. We cannot exclude the possibility that the effects of THPA on cytokines, adhesion molecules and eNOS are secondary to the changes of lipid composition or antioxidative effect.

In addition, we did not observe any dose-responsive effect of THPA on the development of AS, so in the next study, it is necessary to reduce the dosage and try to get the effective minimum dose of THPA to exert anti-atherosclerotic effects in a murine model of AS. Besides, a drug combination study might also be required to determine whether the minimum dose of THPA could enhance the antiatherosclerotic effects of statins.

In conclusion, our data show that *in vivo* administration of the synthesized drug THPA effectively attenuates atherosclerotic lesion formation by targeting several pathways involved in atherogenesis. Elevated plasma apoA-I levels and plasma LCAT activities, decreased plasma PLTP activities, the stimulation of reverse cholesterol transporter gene expression, improved endothelial function, reduced vascular inflammation and the antioxidant effects may all contribute to these beneficial effects of THPA. Our findings provide a novel insight into the antiatherosclerotic effects of THPA and suggest that THPA administration may thus represent a useful therapeutic strategy for AS.

Author contributions: ZZ participated in the design of the studies, analysis of the data and review of the manuscript. GS carried out the study design, data collection and analysis, drafted the manuscript and revised the manuscript. HT performed aorta lesion analysis. YY, XT, JL and SY performed animal studies. TL conducted the PLTP activity analysis. SQ was responsible for the study design, funding, data analysis and manuscript draft. All authors read and approved the final manuscript. ZZ and GS contributed equally to this study.

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REFERENCES

1 Libby P. Inflammation in atherosclerosis. *Nature* 2002;**420**:868–74
2 Smith SC Jr. Review of recent clinical trials of lipid lowering in coronary artery disease. *Am J Cardiol* 1997;**80**:10H–3H
3 Kim HG, Shrestha B, Lim SY, Yoon DH, Chang WC, Shin DJ, Han SK, Park SM, Park JH, Park HI, Sung JM, Jang Y, Chung N, Hwang KC, Kim TW. Cordycepin inhibits lipopolysaccharide-induced inflammation by the suppression of NF-kappaB through Akt and p38 inhibition in RAW 264.7 macrophage cells. *Eur J Pharmacol* 2006;**545**:192–9
4 Shin S, Lee S, Kwon J, Moon S, Lee CK, Cho K, Ha NJ, Kim K. Cordycepin suppresses expression of diabetes regulating genes by inhibition of lipopolysaccharide-induced inflammation in macrophages. *Immune Netw* 2009;**9**:98–105
5 Cho HJ, Cho JY, Rhee MH, Kim HS, Lee HS, Park HJ. Inhibitory effects of cordycepin (3′-deoxyadenosine), a component of *Cordyceps militaris*, on human platelet aggregation induced by thapsigargin. *J Microbiol Biotechnol* 2007;**17**:1134–8
6 Guo P, Kai Q, Gao J, Lian ZQ, Wu CM, Wu CA, Zhu HB. Cordycepin prevents hyperlipidemia in hamsters fed a high-fat diet via activation of AMP-activated protein kinase. *J Pharmacol Sci* 2010;**113**:395–403
7 Won KJ, Lee SC, Lee CK, Lee HM, Lee SH, Fang Z, Choi OB, Jin M, Kim J, Park T, Choi WS, Kim SK, Kim B. Cordycepin attenuates neointimal formation by inhibiting reactive oxygen species-mediated responses in vascular smooth muscle cells in rats. *J Pharmacol Sci* 2009;**109**:403–12
8 Lian Z, Li Y, Gao J, Qu K, Li J, Hao L, Wu S, Zhu H. A novel AMPK activator, WS070117, improves lipid metabolism discords in hamsters and HepG2 cells. *Lipids Health Dis* 2011;**10**:67
9 Ly H, Francone OL, Fielding CJ, Shigenaga JK, Moser AH, Grunfeld C, Feingold KR. Endotoxin and TNF lead to reduced plasma LCAT activity and decreased hepatic LCAT mRNA levels in Syrian hamsters. *J Lipid Res* 1995;**36**:1254–63
10 Schlitt A, Bickel C, Thumma P, Blankenberg S, Rupprecht HJ, Meyer J, Jiang XC. High plasma phospholipid transfer protein levels as a risk factor for coronary artery disease. *Arterioscler Thromb Vasc Biol* 2003;**23**:1857–62
11 Ayaori M, Sawada S, Yonemura A, Iwamoto N, Ogura M, Tanaka N, Nakaya K, Kusuhara M, Nakamura H, Ohsuzu F. Glucocorticoid receptor regulates ATP-binding cassette transporter-A1 expression and apolipoprotein-mediated cholesterol efflux from macrophages. *Arterioscler Thromb Vasc Biol* 2006;**26**:163–8
12 Gordon DJ, Rifkind BM. High-density lipoprotein – the clinical implications of recent studies. *N Engl J Med* 1989;**321**:1311–6
13 Navab M, Anantharamaiah GM, Reddy ST, Van Lenten BJ, Buga GM, Fogelman AM. Peptide mimetics of apolipoproteins improve HDL function. *J Clin Lipidol* 2007;**1**:142–7
14 Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ Res* 2005;**96**:1221–32
15 Jiang XC, Qin S, Qiao C, Kawano K, Lin M, Skold A, Xiao X, Tall AR. Apolipoprotein B secretion and atherosclerosis are decreased in mice with phospholipid-transfer protein deficiency. *Nat Med* 2001;**7**:847–52
16 Jiang XC, Tall AR, Qin S, Lin M, Schneider M, Lalanne F, Deckert V, Desrumaux C, Athias A, Witztum JL, Lagrost L. Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E. *J Biol Chem* 2002;**277**:31850–6
17 Tu AY, Nishida HI, Nishida T. High density lipoprotein conversion mediated by human plasma phospholipid transfer protein. *J Biol Chem* 1993;**268**:23098–105
18 Jauhiainen M, Metso J, Pahlman R, Blomqvist S, van Tol A, Ehnholm C. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J Biol Chem* 1993;**268**:4032–6
19 Murdoch SJ, Carr MC, Hokanson JE, Brunzell JD, Albers JJ. PLTP activity in premenopausal women. Relationship with lipoprotein lipase, HDL, LDL, body fat, and insulin resistance. *J Lipid Res* 2000;**41**:237–44
20 Cheung MC, Wolfbauer G, Kennedy H, Brown BG, Albers JJ. Plasma phospholipid transfer protein activity in patients with low HDL and cardiovascular disease treated with simvastatin and niacin. *Biochim Biophys Acta* 2001;**1537**:117–24
21 Sahoo D, Trischuk TC, Chan T, Drover VA, Ho S, Chimini G, Agellon LB, Agnihotri R, Francis GA, Lehner R. ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes. *J Lipid Res* 2004;**45**:1122–31
22 Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, Shear CL, Revkin JH, Buhr KA, Fisher MR, Tall AR, Brewer B. Effects of torcetrapib in patients at high risk for coronary events. *N Engl J Med* 2007;**357**:2109–22
23 Wang N, Silver DL, Thiele C, Tall AR. ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J Biol Chem* 2001;**276**:23742–7
24 Kennedy MA, Barrera GC, Nakamura K, Baldan A, Tarr P, Fishbein MC, Frank J, Francone OL, Edwards PA. ABCG1 has a critical role in

mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab* 2005;**1**:121–31
25 Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc Natl Acad Sci USA* 2004;**101**:9774–9
26 Ji Y, Jian B, Wang N, Sun Y, Moya ML, Phillips MC, Rothblat GH, Swaney JB, Tall AR. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J Biol Chem* 1997;**272**:20982–5
27 Kuivenhoven JA, Pritchard H, Hill J, Frohlich J, Assmann G, Kastelein J. The molecular pathology of lecithin:cholesterol acyltransferase (LCAT) deficiency syndromes. *J Lipid Res* 1997;**38**:191–205
28 Ng DS. Insight into the role of LCAT from mouse models. *Rev Endocr Metab Disord* 2004;**5**:311–8
29 Jonas A. Regulation of lecithin cholesterol acyltransferase activity. *Prog Lipid Res* 1998;**37**:209–34
30 Zerfaoui M, Suzuki Y, Naura AS, Hans CP, Nichols C, Boulares AH. Nuclear translocation of p65 NF-kappaB is sufficient for VCAM-1, but not ICAM-1, expression in TNF-stimulated smooth muscle cells: differential requirement for PARP-1 expression and interaction. *Cell Signal* 2008;**20**:186–94
31 Li YD, Ye BQ, Zheng SX, Wang JT, Wang JG, Chen M, Liu JG, Pei XH, Wang LJ, Lin ZX, Gupta K, Mackman N, Slungaard A, Key NS, Geng JG. NF-kappaB transcription factor p50 critically regulates tissue factor in deep vein thrombosis. *J Biol Chem* 2009;**284**:4473–83
32 Agusti A, Morla M, Sauleda J, Saus C, Busquets X. NF-kappaB activation and iNOS upregulation in skeletal muscle of patients with COPD and low body weight. *Thorax* 2004;**59**:483–7

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