RESEARCH ARTICLE

Long-term curcumin administration protects against atherosclerosis via hepatic regulation of lipoprotein cholesterol metabolism

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Scope: Atherosclerosis is a major cause of cardiovascular disease caused by high cholesterol. Stains are widely prescribed to lower cholesterol levels, but natural dietary compounds may also be effective. Therefore, we studied the effect of the natural dietary compound curcumin on atherosclerosis and its underlying mechanisms based on plasma and hepatic lipid metabolism.

Methods and results: LDLR$^{-/-}$ mice were fed a high-cholesterol diet and treated with curcumin, lovastatin or control ($n = 10$ per group) for 18 wk. Aortic arch sections revealed curcumin ameliorated early atherosclerotic lesions, lipid infiltration, ICAM-1 and VCAM-1 localization, similar to lovastatin treatment. Furthermore, curcumin lowered plasma cholesterol, triglycerides, LDL cholesterol and Apo B levels as well as CETP activity, while curcumin increased plasma HDL cholesterol and liver Apo A-I expression, similar to lovastatin treatment. Curcumin caused transcriptional inhibition of HMG-CoA reductase, independent of ACAT1 and ACAT2 expression. Hepatic PPARα and LXRα expression was upregulated by curcumin treatment. Hepatic complement factor D (Cfd) and systemic CRP levels, markers of immune complement pathway activation, were significantly reduced by curcumin treatment.

Conclusion: Long-term curcumin treatment lowers plasma and hepatic cholesterol and suppresses early atherosclerotic lesions comparable to the protective effects of lovastatin. The anti-atherogenic effect of curcumin is mediated via multiple mechanisms including altered lipid, cholesterol and immune gene expression.

Keywords:
Atherosclerosis / Curcumin / Gene expression / HMG-CoA / Lovastatin

1 Introduction

Atherosclerosis caused by the accumulation of lipids in the arterial wall is a major factor leading to heart disease and stroke [1]. The etiology of atherosclerosis is complex due to multiple genetic and environmental risk factors. Atherosclerosis occurs with age, but excessive dietary lipid intake, resulting in elevated plasma cholesterol or triglyceride levels may accelerate the atherogenic process [1]. Currently, statins that were first discovered as a natural metabolite in *Aspergillus terreus* are the most widely prescribed drug to lower plasma cholesterol levels [2]. However, there is widespread

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Abbreviations: ACAT, acyl-CoA/cholesterol acyltransferase; **Apo A-I,** apolipoprotein A-I; **Apo B,** apolipoprotein B; **CETP,** cholesteryl ester transferase; **Cfd,** complement factor D; **FAS,** fatty acid synthesis; **HDL-C,** HDL-cholesterol; **HMG-CoA,** 3-hydroxy-3-methyl-glutaryl-co-enzyme A reductase; **HMGR,** 3-hydroxy-3-methylglutaryl-co-enzyme A reductase; **hsCRP,** high-sensitivity C-reactive protein; **ICAM-1,** inter-cellular adhesion molecule 1; **LDL-C,** LDL-cholesterol; **LXRα,** liver X receptor α; **PPARα,** peroxisome proliferator-activated receptor α; **total-C,** total cholesterol; **VCAM-1,** vascular cell adhesion molecule 1

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interest in establishing alternative non-pharmacological ways to manage cholesterol based on natural dietary compounds, which may prove to be more effective than statins for reducing atherosclerosis risk [3, 4].

Curcumin is a natural ingredient found in turmeric, which comes from the *Curcuma longa* plant [3]. There is accumulating evidence on the effects of curcumin in a variety of pathological conditions including cancer, inflammation, obesity and cardiovascular disease [4]. Over three decades ago, curcumin was first reported to lower hepatic cholesterol and modify plasma lipoprotein levels [5]. Subsequently, curcumin has been widely reported to protect against hypercholesterolemia in animals fed an atherogenic diet [6–13]. The evidence that curcumin lowers triglyceride levels is equivocal, some studies demonstrate that curcumin results in hypotriglyceridemia in liver but not plasma [13–15]. While a few studies suggest that curcumin neither lowers cholesterol nor triglyceride levels, however, these findings may be attributable to either short treatment duration [14] or administration of insufficient fat/cholesterol inducing diets [15–17]. While the in vivo effects of curcumin on plasma lipids have been extensively reported, the mechanisms behind the lipid lowering effects of curcumin are not fully understood. Furthermore, while the lipid-lowering effects of curcumin strongly suggest an anti-atherogenic effect, the direct effect of curcumin on atherosclerotic lesion development is unclear from past studies [11, 16, 18].

In the present study, we firstly examined the long-term effects of curcumin compared with lovastatin treatment on atherosclerotic lesion development in $LDLR^{-/-}$ mice fed a high-cholesterol diet. Secondly, we examined whether the long-term lipid lowering effects of curcumin are mediated via regulation of cholesterol metabolism, lipid metabolism or inflammation markers in liver or plasma.

2 Materials and methods

2.1 Animals and diets

Thirty-four-wk-old male $LDLR^{-/-}$ mice (background strain C57BL/6J) were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). After 1 wk of adaptation, mice were randomly divided into three groups ($n = 10$ per group). All mice were fed an atherogenic diet for 18 wk based on the semipurified AIN-76 diet, which contained 1% cholesterol, 17.4% cocoa butter, 0.1% sodium cholate, 2.8% soy oil and either 0.02% w/w lovastatin (LV, Tokyo Chemical) or 0.02% w/w curcumin (CC, Sigma Chemical). Soy oil was included to provide sufficient essential fatty acid (EFA) content. There was no difference in body weight gain (Supporting Information Fig. 1) or food intake (Supporting Information Table 1) between curcumin and placebo-treated mice.

Venous blood was drawn from the tail vein every 3 wk for the determination of the plasma total cholesterol (total-C) and triglyceride concentration. After 18 wk, blood was drawn from the inferior vena cava for plasma lipid profile analysis, and then mice were anesthetized and sacrificed. Livers were immediately removed, rinsed, weighed and then immediately frozen in liquid nitrogen and stored at –70°C until analysis. The study protocol was approved by the Ethics Committee for animal studies (knu-2010-1-37) at Kyungpook National University, Republic of Korea.

2.2 Histological staining and immunohistochemistry of the aorta arch

Each aortic arch was removed and wrapped with saline-soaked gauze after dissecting out any connective tissues. Then each aortic arch was fixed in 10% paraformaldehyde/PBS, embedded in paraffin and stained with hematoxylin and eosin (H&E). Another section of the aortic arch was cryosectioned and stained with Oil-Red O solution to highlight any lipid infiltration. For immunohistochemistry, the aortic arch was cryostat sectioned, fixed in hydrogen peroxide and washed in citrate buffer (pH 6.0). These sections were treated with blocking reagent (Ultra Tech HRP, USA) to prevent non-specific binding and incubated with monoclonal antibodies against vascular cell adhesion molecule 1 (VCAM-1) or inter-cellular adhesion molecule 1 (ICAM-1; Santa Cruz Biotech). Antibody reactivity was detected by using HRP-conjugated biotin–streptavidin complexes and developed with diaminobenzidine tetrahydrochloride as the substrate.

2.3 Plasma and hepatic lipid analysis

Plasma lipids [total-C, triglyceride and HDL-cholesterol (HDL-C)] concentrations were determined using enzymatic kits (Asan, Seoul, Republic of Korea). The levels of plasma apolipoprotein A-I (Apo A-I) and apolipoprotein B (Apo B) were measured by immunoassay (Nitto Boseki, Tokyo, Japan). Hepatic tissue was homogenized in 20 mM potassium phosphate buffer (pH 7.4) and lipids were extracted using chloroform/methanol (1:1, v/v) solution. Triton X-100 and a sodium cholate solution were added to the dissolved lipids for emulsification. Hepatic cholesterol and triglycerides were analyzed with the same enzymatic kit as used for plasma lipid analysis. ELISA kits were used to determine plasma high-sensitivity C-reactive protein (hsCRP; Kamiya Biomedical, WA, USA), adipsin (Millipore, MA, USA) and cholesteryl ester transferase (CETP; BioVision, CA, USA).

2.4 Determination of hepatic HMG-CoA and ACAT activity

Hepatic microsomes were prepared according to established methods [19]. Briefly, liver tissue (2 g) was homogenized in 4 mL ice-cold buffer (pH 7.0) containing 0.1 M triethanolamine,

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0.02 M EDTA and 2 mM dithiothreitol. Homogenates were centrifuged twice, at 10 000 × *g*, then 12 000 × *g* for 15 min at 4°C. Afterwards, the supernatants were ultra-centrifuged twice at 100 000 × *g* for 60 min at 4°C. The resulting microsomal pellets were redissolved in 1 mL of homogenization buffer without DTT. After the determination of protein concentrations [20], microsomes were analyzed for the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) and acyl-CoA/cholesterol acyltransferase (ACAT). Microsomal HMGR activity was measured with [^{14}C]-3-hydroxy-3-methyl-glutaryl-co-enzyme A reductase (HMG-CoA) as the substrate using previously established methods [21]. Microsomal ACAT activities were determined using [^{14}C]-oleoyl CoA as described previously [22].

2.5 RNA isolation and quality control

Total RNA was extracted from liver using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. DNase digestion was used to remove any DNA contamination, and RNA was re-precipitated in ethanol to ensure no phenol contamination. For quality control, RNA purity and integrity were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA was stored at –70°C prior to further analysis by RT-qPCR.

2.6 RT-qPCR analysis

Total RNA (1 μg) was reverse transcribed into cDNA using the QuantiTect® reverse transcription kit (Qiagen, Germany). Then mRNA expression was quantified by real-time quantitative PCR, using the QuantiTect® SYBR green PCR kit (Qiagen) on the CFX96TM real-time PCR system (Bio-Rad, UK). Gene-specific mouse primers (Supporting Information Table 2) were used to detect Apo AI and liver X receptor α (LXRα), HMGR, acyl-coenzyme A/cholesterol acyltransferase 1 (ACAT1), acyl-coenzyme A acyltransferase 2 (ACAT2), complement factor D (Cfd), fatty acid synthesis (FAS) and peroxisome proliferator-activated receptor α (PPARα). Cycle thresholds were determined based on SYBR green emission intensity during the exponential phase. C_t data were normalized using GAPDH, which was stably expressed across all experimental groups. Relative gene expression was calculated using the $-2^{\Delta\Delta C_t}$ method [23].

2.7 Statistical analysis

Differences between control, lovastatin and curcumin-treated mice were examined by one-way ANOVA using SPSS (SPSS, Chicago, IL, USA). Any significant differences were analyzed further using Duncan's multiple-range test. Statistical significance was considered acceptable at $p<0.05$. All data are presented as mean ± standard error.

3 Results

3.1 Effect of curcumin on the development of atherosclerotic lesions

Histopathological differences between untreated, lovastatin and curcumin-treated LDLR$^{-/-}$ mice fed an atherogenic diet for 18 wk were prominent in the ascending aorta. In the untreated LDLR$^{-/-}$ mice, an atherosclerotic lesion was observable at the edge of the aortic dissection that extended over the inside of the aortic arch (Fig. 1A). However, aortic arch sections of lovastatin and curcumin-treated mice showed no visible intimal lesions. Oil-Red-O staining revealed lipid infiltration into the aortic lesions of untreated LDLR$^{-/-}$ mice, but not their counterparts treated with either lovastatin or curcumin (Fig. 1B). Aortic sections were stained for ICAM-1 and VCAM-1. The aortas of untreated LDLR$^{-/-}$ mice contained a fatty plaque that was readily visible upon ICAM-1 exposure (Fig. 1C). Immunohistochemistry also confirmed the presence of abundant VCAM-1 within the aortic fatty plaque of untreated LDLR$^{-/-}$ mice (Fig. 1D) but was not present in curcumin or lovastatin-treated mice.

3.2 Effect of curcumin on plasma cholesterol and triglyceride levels

Concentration of lower plasma total cholesterol was evident in the curcumin-treated mice after 6 wk, similar to the cholesterol lowering effect of lovastatin, compared with untreated mice (50.5 ± 2.3 and 54.8 ± 0.6 versus 58.2 ± 1.4 mmol/L; $p<0.05$; Fig. 2A). Plasma total cholesterol accumulation was consistently suppressed after 12 wk of curcumin or lovastatin treatment until the end of the study. At 18 wk, plasma total cholesterol level was significantly lower in the curcumin and lovastatin-treated mice, compared with the untreated controls (45.1 ± 0.5 and 44.1 ± 0.4 versus 49.2 ± 0.2 mmol/L; $p<0.05$; Fig. 2A). Similarly, plasma triglyceride levels were lower in the curcumin and lovastatin-treated mice after 6 wk and consistently suppressed until the end of the study (Fig. 2B). At 18 wk, plasma triglyceride levels were significantly lower in the curcumin and lovastatin-treated mice, compared with untreated controls (4.0 ± 0.2 and 4.4 ± 0.2 versus 5.2 ± 0.3 mmol/L; $p<0.05$; Fig. 2B)

3.3 Effect of curcumin on plasma lipoprotein and regulation of cholesterol ester transferase activity

Plasma analysis of lipoproteins revealed that HDL-C was significantly increased in the curcumin and lovastatin-treated mice compared with untreated mice (0.51 ± 0.03 and 0.41 ± 0.03 versus 0.39 ± 0.03 mmol/L; $p<0.05$,

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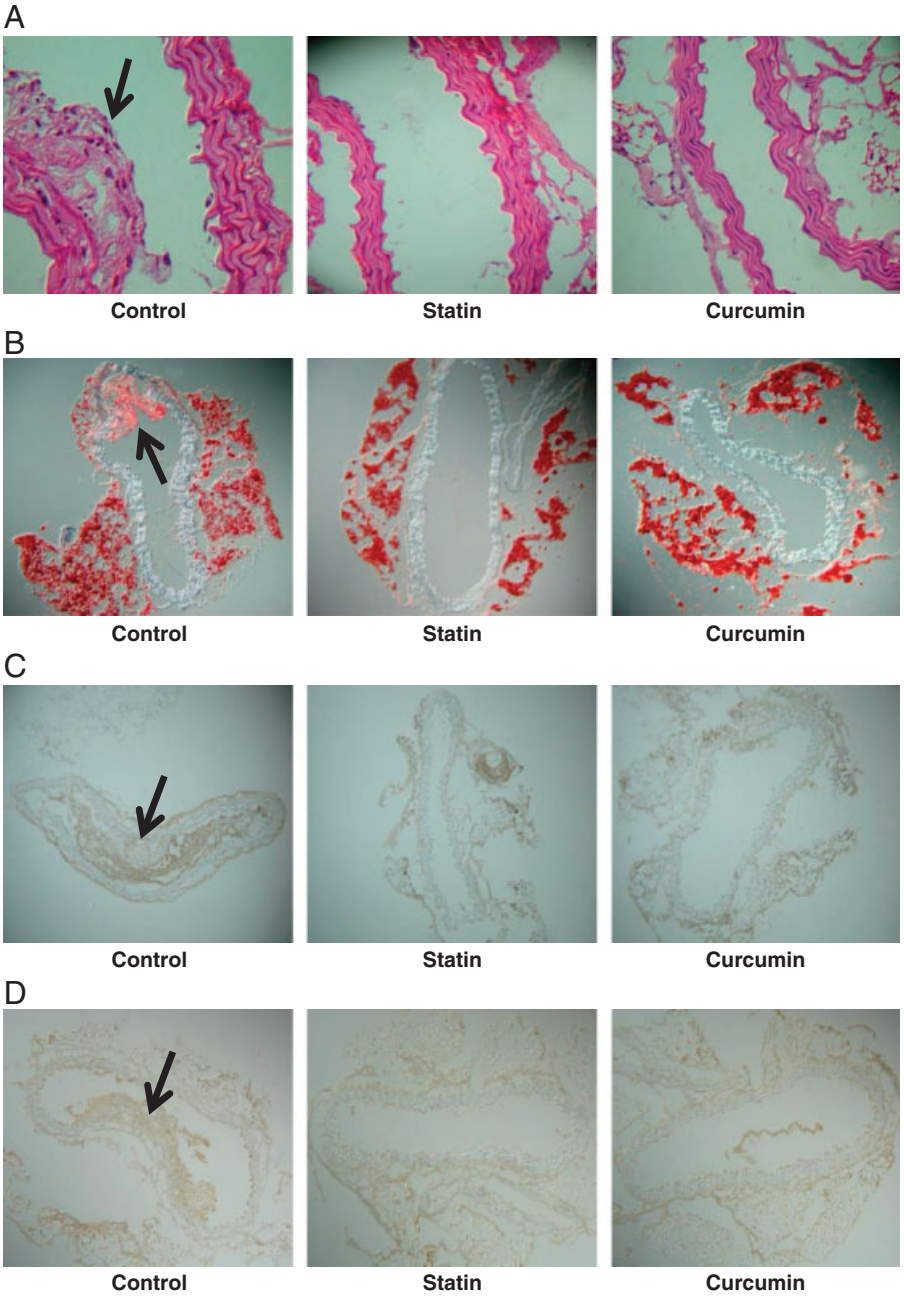

Figure 1. Histological and immunohistochemical staining of aortic arch from LDLR$^{-/-}$ mice fed an atherogenic diet with curcumin or lovastatin treatment for 18 wk. (A) H&E stained transverse section of aortic arch (400× magnification). (B) Oil-Red O stained cryosection of aortic arch (200× magnification). (C) Immunostained transverse section of aortic arch with anti-ICAM-1 (100× magnification). (D) Immunostained transverse section of aortic arch with anti-VCAM-1 (100× magnification). Arrows indicate atherosclerotic lesion and fatty streak.

Fig. 3A). Apo A-I, a major component of HDL-C, was unchanged by curcumin or lovastatin treatment (Fig. 3B). We measured Apo AI mRNA expression in liver tissue and found hepatic Apo AI expression was significantly upregulated in curcumin and lovastatin-treated mice ($p<0.05$, Fig. 3C).

Plasma LDL-cholesterol (LDL-C) was significantly decreased in the curcumin and lovastatin-treated mice compared with untreated mice (43.1 ± 0.7 and 43.1 ± 0.6 versus 47.9 ± 0.2 mmol/L; $p<0.05$, Fig. 3D). Similarly, Apo B, the main apolipoprotein found in LDL-C, was significantly lower in curcumin and lovastatin-treated mice (47.6 ± 0.9 and 48.2 ± 1.4 versus 53.0 ± 0.8 mg/dL; $p<0.05$, Fig. 3E). We measured CETP activity, which facilitates the transport of cholesteryl esters and triglycerides between HDL-C and lower density lipoprotein. Curcumin and lovastatin treatment both significantly inhibited CETP activity compared with untreated mice (16.6 ± 0.2 and 17.0 ± 0.4 versus 18.0 ± 0.2 μmol/μL/h; $p<0.05$, Fig. 3F). Biomarkers of atherosclerosis including the atherogenic index, % of HDL-C/total-C and Apo B/Apo A-I ratio were all improved by curcumin treatment, and furthermore curcumin appeared to be more effective than lovastatin treatment in some cases (Table 1).

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3.4 Effect of curcumin on liver cholesterol and the regulation of cholesterol metabolizing

Next, we measured the changes in liver cholesterol metabolism pathways, including cholesterol biosynthesis and esterification to further examine the mechanism underlying the plasma lipid lowering effect of curcumin. Firstly, we observed curcumin and lovastatin treatment both reduced hepatic cholesterol accumulation in liver; however, curcumin was significantly more effective than lovastatin (1.60 ± 0.01 versus 1.73 ± 0.01 versus 1.88 ± 0.04 mmol/g liver; $p < 0.05$; Fig. 4A). Next, we examined whether this was due to an alteration in cholesterol biosynthesis. Curcumin treatment significantly downregulated HMGR gene expression in liver compared with untreated controls (Fig. 4B). Remarkably, curcumin was more effective than lovastatin at inhibiting HMGR gene expression ($40 \pm 2\%$ versus $28 \pm 1\%$; $p < 0.05$; Fig. 4B). The inhibition of HMGR gene expression was consistent with decreased HMG-CoA enzyme activity (Fig. 4C), suggesting curcumin suppressed HMG-CoA activity via transcriptional inhibition. We also examined ACAT1 and ACAT2 expression, which encode acyl-CoA/cholesterol acetyl transferases that regulate the synthesis of cholesteryl esters. Neither, ACAT1 or ACAT2 expression was affected by curcumin or lovastatin treatment (Fig. 4D and E). Consistent with the lack of transcriptional regulation of ACAT gene expression, we observed that hepatic ACAT activity was unchanged by curcumin or lovastatin treatment (Fig. 4F).

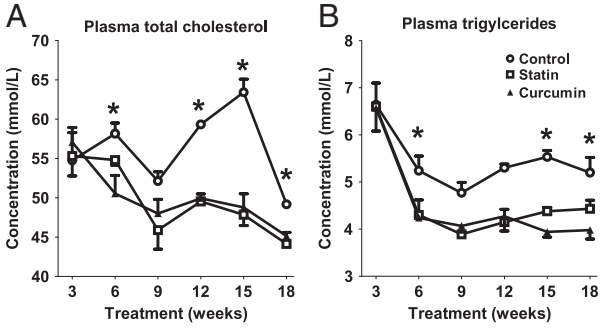

Figure 2. Time course of changes in (A) plasma total cholesterol and (B) triglyceride concentrations in LDLR$^{-/-}$ mice fed an atherogenic diet with curcumin or lovastatin treatment for 18 wk. Data are shown as mean $\pm$ SEM. Asterisk indicates significant differences between curcumin- and lovastatin-treated mice compared with untreated controls at $p < 0.05$.

3.5 Effect of curcumin on hepatic triglycerides and the regulation of hepatic lipid metabolism genes

Next we examined whether curcumin altered hepatic triglyceride accumulation and nuclear transcriptional factors which regulate lipid metabolism genes. Firstly,

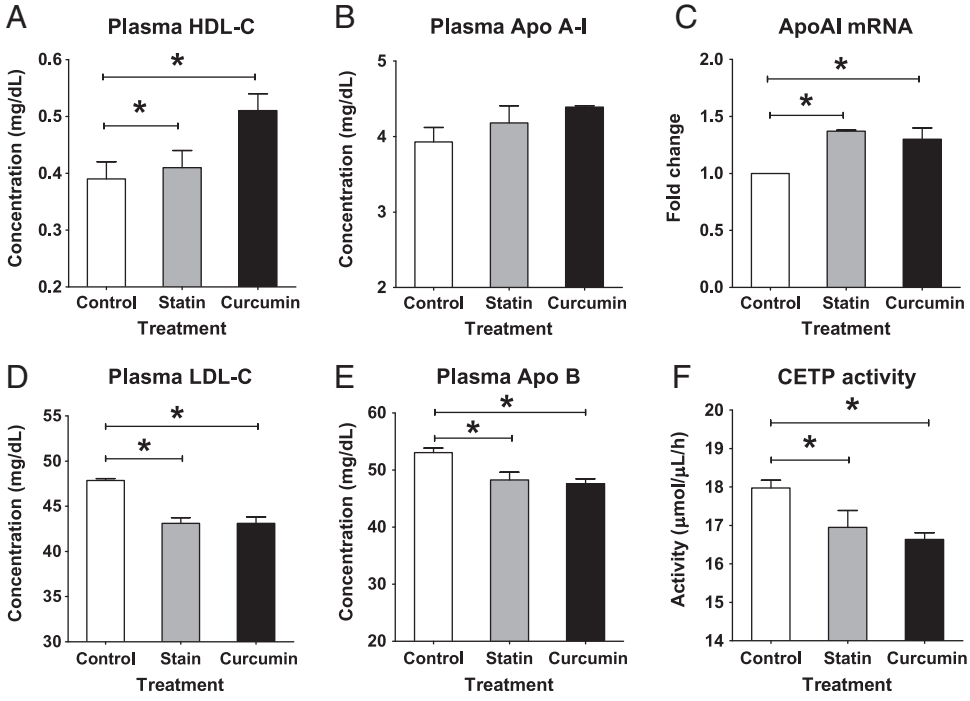

Figure 3. Effect of curcumin or lovastatin treatment on lipoprotein cholesterol concentration and cholesterol ester transfer protein activity in LDLR$^{-/-}$ mice fed an atherogenic diet with curcumin or lovastatin treatment for 18 wk. (A) Plasma HDL-C. (B) Plasma Apo A-I. (C) Hepatic Apo AI mRNA. (D) Plasma LDL-C. (E) Plasma ApoB. (F) Plasma cholesterol ester transferase (CETP) activity. Data are shown as mean $\pm$ SEM. Asterisk indicates significant differences between groups at $p < 0.05$.

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Table 1. Effects of curcumin on surrogate plasma atherogenic markers in LDLR$^{-/-}$ mice fed an atherogenic diet with curcumin or lovastatin treatment for 18 wk

	Control	Lovastatin	Curcumin
Atherogenic index	125.94 ± 11.48[a]	105.22 ± 9.19[ab]	87.37 ± 4.24[b]
HDL-C/total-C (%)	0.81 ± 0.07[a]	0.94 ± 0.08[ab]	1.13 ± 0.08[b]
Apo B/Apo AI ratio	13.46 ± 0.35[a]	11.12 ± 0.22[b]	10.97 ± 0.16[b]

Data are means ± SEM ($n = 10$ per group).
[ab]Means in the same row not sharing a common superscript are significantly different among groups at $p < 0.05$.
HDL-C, high-density lipoprotein cholesterol; AI, (total-C/HDL-C)/HDL-C; Apo, apolipoprotein.

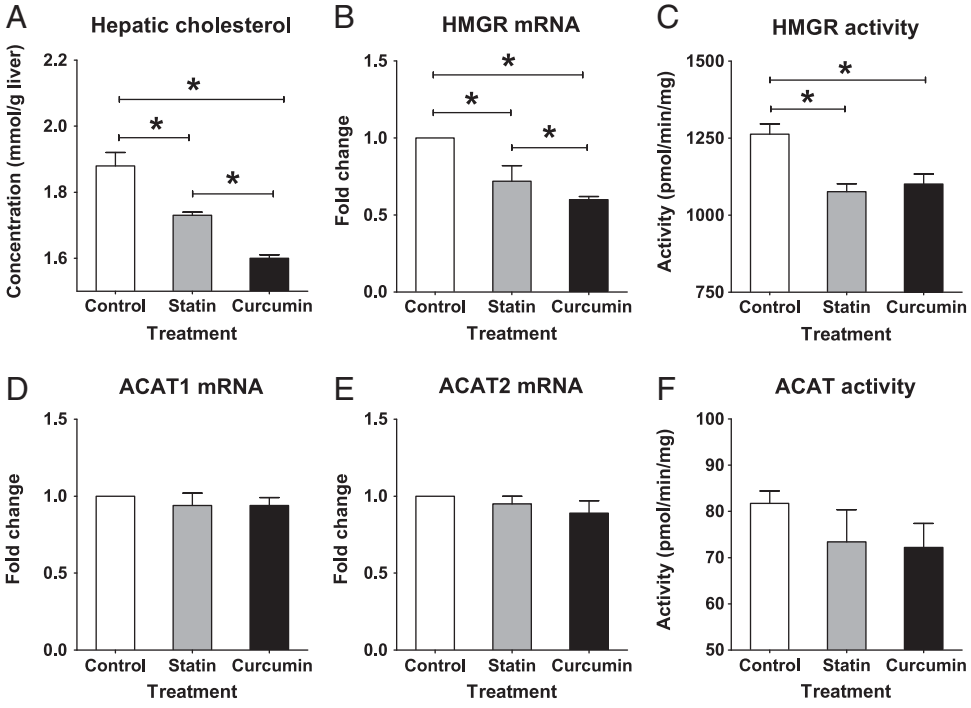

Figure 4. Effect of curcumin or lovastatin treatment on hepatic cholesterol and cholesterol regulating enzymes in LDLR$^{-/-}$ mice fed an atherogenic diet with curcumin or lovastatin treatment for 18 wk. (A) Hepatic cholesterol. (B) HMGR gene expression. (C) HMGR activity. (D) ACAT1 gene expression. (E) ACAT2 gene expression and (F) ACAT activity. Data are shown as mean ± SEM. Asterisk indicates significant differences between groups at $p < 0.05$. HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; ACAT, acyl-CoA cholesterol acyltransferase.

we observed both curcumin and lovastatin treatment significantly suppressed hepatic triglyceride accumulation (0.98 ± 0.03 and 0.94 ± 0.03 versus 1.11 ± 0.05 mmol/g liver; $p < 0.05$; Fig. 5A). We observed no change in FAS expression, which encodes the fatty acid synthase enzyme, following curcumin treatment, but FAS expression was decreased by lovastatin treatment (Fig. 5B). Curcumin and lovastatin treatment increased PPARα expression ~30%, which is nuclear receptor factor reported to have widespread effects on genes associated with mitochondrial fatty acid oxidation (Fig. 5C). Expression of LXRα, another nuclear receptor factor that regulates lipid synthesis, was also significantly upregulated by curcumin and to a lesser extent lovastatin treatment (88 ± 3% versus 47 ± 5%; $p < 0.05$; Fig. 5D).

3.6 Effect of curcumin on systematic inflammation and complement factor activation

Both curcumin and lovastatin have been widely reported to have anti-inflammatory effects; consistent with these reports we observed that curcumin and lovastatin treatment significantly lowered plasma hsCRP levels (4.0 ± 0.1 and 3.8 ± 0.1 versus 4.6 ± 0.3 ng/mL; $p < 0.05$; Fig. 6A). We also measured Cfd (also known as adipsin), which plays a role in the

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complement immune system pathway. We found plasma adipsin levels were only lowered by lovastatin but not by curcumin treatment (Fig. 6B). However, in liver both curcumin and lovastatin significantly inhibited Cfd gene expression (Fig. 6C), indicating reduced activation of the complement immune system pathway.

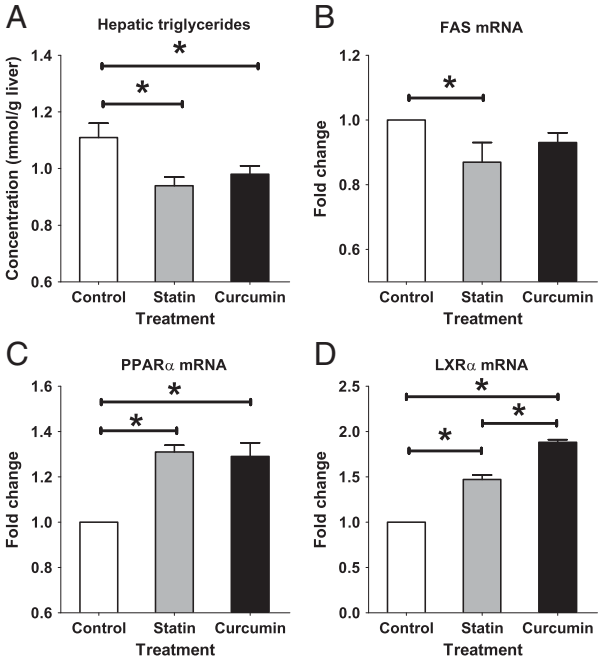

Figure 5. Effect of curcumin or lovastatin treatment on hepatic triglycerides and transcriptional regulators of lipid metabolism in LDLR$^{-/-}$ mice fed an atherogenic diet with curcumin or lovastatin treatment for 18 wk. (A) Hepatic triglycerides, (B) FAS gene expression, (C) PPARα gene expression and (D) LXRα gene expression. Data are shown as mean ± SEM. Asterisk indicates significant differences between groups at $p < 0.05$. FAS, fatty acid synthesis; PPARα, peroxisome proliferator-activated receptor α; LXRα, liver X receptor α.

4 Discussion

The cholesterol lowering effects of curcumin were first reported several decades ago [5], but the mechanisms underlying the in vivo action of curcumin are still not fully understood [3]. In the present study, we used a diet-induced atherogenic model to first establish the long-term effect of curcumin on atherosclerosis and secondly to examine the mechanisms underlying the in vivo effects of curcumin treatment in LDLR$^{-/-}$ mice (Fig. 7).

4.1 Curcumin suppresses the development of early atherosclerotic lesions and decreases surrogate plasma atherogenic markers induced by a high-cholesterol diet

Accumulation of lipids and inflammatory cells in the wall of major arteries over time is a major hallmark of atherosclerosis [1]. Early atherosclerotic lesion formation, infiltration of lipids, as well as localization of ICAM-1 and VCAM-1 to the atherosclerotic plaque was evident in the aortic arch of LDLR$^{-/-}$ mice fed a high cholesterol diet for 18 wk. VCAM-1 and ICAM-1 are inflammatory adhesion molecules, which mediate monocyte attachment to the arterial vascular endothelium [24]. VCAM-1 and ICAM-1 are predominantly expressed by the endothelium in early lesions [25]. However, curcumin- and lovastatin-treated mice showed no evidence of early atherosclerotic lesions or lipid infiltration in the aortic arch compared with untreated mice. These observations suggest that curcumin can effectively suppress early atherosclerotic lesion formation in LDLR$^{-/-}$ mice fed an atherogenic diet.

Past studies have been less equivocal with only minimal reduction or no protective effect of curcumin on early atherosclerotic lesion formation reported in Apo E/LDLR$^{-/-}$

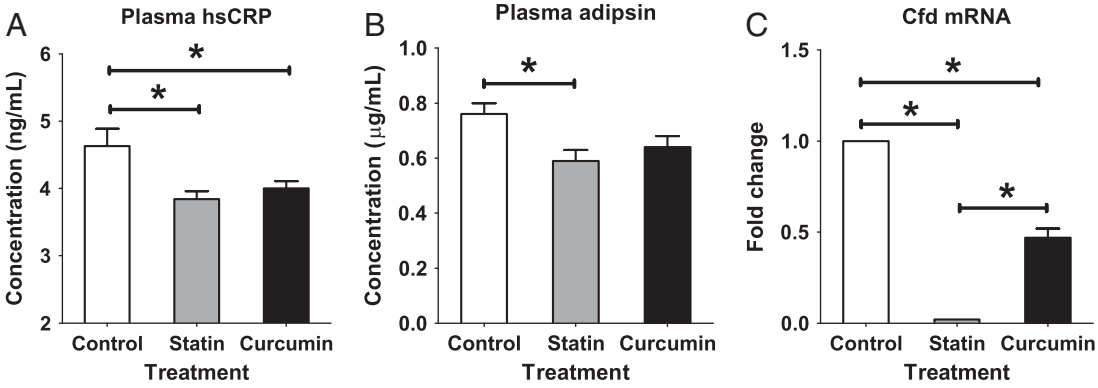

Figure 6. Effect of curcumin or lovastatin treatment on plasma hsCRP and adipsin concentration and hepatic Cfd mRNA expression in LDLR$^{-/-}$ mice fed an atherogenic diet with curcumin or lovastatin treatment for 18 wk. (A) Plasma hsCRP, (B) plasma adipsin and (C) hepatic Cfd mRNA expression. Data are shown as mean ± SEM. Asterisk indicates significant differences between groups at $p < 0.05$. hsCRP, high sensitivity C-reactive protein; cfd, complement factor D.

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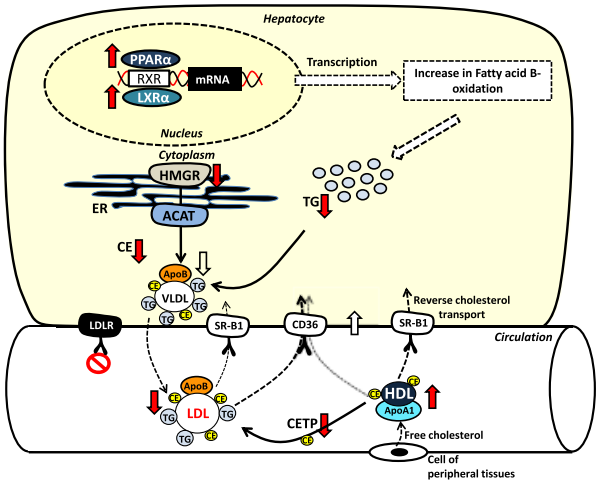

Figure 7. Proposed summary of the lipid lowering action and anti-atherogenic property of curcumin in LDLR$^{-/-}$ mice fed an atherogenic diet. Curcumin upregulates hepatic PPARα and LXRα expression, which activates transcription of fatty acid oxidation and cholesterol transport related genes, leading to lower hepatic cholesterol and triglycerides. Curcumin inhibits HMGR gene expression and its activity thereby decreasing cholesterol biosynthesis but has no effect on ACAT. Accordingly, VLDL formation and secretion may be reduced by curcumin treatment. Curcumin led to lower plasma LDL-C via reduction of CE transfer from HDL and/or enhanced clearance of plasma LDL-C which can be mediated by hepatic CD36 and SR-B1 under condition of LDLR deficiency [52]. Curcumin-induced reduction of CE transfer between lipoproteins due to CETP inhibition results in decreasing LDL-C with simultaneous increasing HDL-C concentration. In addition, the SR-B1 in the liver could increase the flux of reverse cholesterol transport [52]. Abbreviations: ACAT, acyl-CoA/cholesterol acyltransferase; ABCA1, ATP-binding cassette, sub-family A1; CE, cholesteryl esters; CETP, cholesteryl ester transferase; ER, endoplasmic reticulum; HMGR, 3-hydroxy-3-methyl-glutaryl-co-enzyme A reductase; RXR, retinoid X receptor; SR-B1, scavenger receptor B1; TG, triglycerides.

mice [16] and rabbits fed an atherogenic diet [11, 18]. However, inconsistent changes in cholesterol or triglycerides reported in these studies suggest that the amount of dietary lipids or duration of diet may have been inadequate to cause hyperlipidemia.

Consistent with the suppression of early aortic lesions, we found surrogate atherogenic indicators including the apo B/apo A-I ratio and the atherogenic index were reduced, while percentage of HDL-C/total-C was improved by curcumin treatment. The prevailing evidence indicates that the changes in these atherogenic indicators translate into lower risk of myocardial infarction, stroke and cardiovascular disease [26]. Importantly, we demonstrate for the first time that curcumin appears to be at least as effective as lovastatin for inhibiting early atherosclerotic lesion development in high cholesterol diet fed mice.

4.2 Long-term curcumin treatment prevents diet induced hypercholesterolemia and hypertriglyceridemia

Next we examined the effect of curcumin on plasma cholesterol and triglyceride levels. The development of atherosclerosis is widely associated with prolonged elevation of plasma cholesterol and triglycerides, which are established cardiovascular disease risk factors in humans [2]. We observed that curcumin significantly lowered plasma cholesterol and triglyceride levels compared with untreated mice over 18 wk. Past studies have reported mixed findings on the effect of curcumin on plasma cholesterol and triglyceride levels. Lower plasma cholesterol is the most universal observation [6–13]. Studies reporting no hypocholesterolemic or hypotriglyceridemic effect of curcumin have been due to either very short duration [14] or used insufficient fat/cholesterol inducing diets [15–17]. In contrast to statins, curcumin is reported to have no protective effect on plasma cholesterol in animals consuming a normal diet [3, 5, 6, 17]. Taken together, these findings indicate that curcumin lowers plasma cholesterol or triglycerides only in the presence of a high fat/cholesterol diet.

4.3 Long-term curcumin alters plasma lipoprotein cholesterol levels and inhibits systemic cholesteryl ester transfer protein activity

Cholesterol loading of the major lipoproteins responsible for the transport of lipids was modulated by curcumin treatment in vivo in LDLR$^{-/-}$ mice fed a high-cholesterol diet over 18 wk. Plasma LDL-C and Apo B levels were lowered by curcumin treatment, similar to lovastatin treatment. Apo B is synthesized and released from the liver, where it forms a 1:1 lipoprotein complex with LDL-C; hence, lower Apo B indicates constitutively lower LDL-C levels. Several studies report that curcumin lowers plasma LDL-C levels in different diet-induced disease models, all with wild-type LDLR [6, 7, 9, 13, 27]. The increased clearance of LDL-C following curcumin and statin treatment in LDLR$^{-/-}$ mice suggests either reduced LDL-C production or that other receptors compensate for the lack of LDLR. Several LDL remnant receptors exist, which can all recognize Apo B containing lipoproteins, including LDLR-related protein (LRP), scavenger receptors and cell surface proteoglycans [28]. Uptake of LDL-C via these alternative LDL remnant receptors could explain the lower Apo-B-associated LDL-C in curcumin-treated LDLR$^{-/-}$ mice.

While lowering LDL-C levels remains a mainstay of current cholesterol management guidelines [29], recent studies indicate increasing HDL-C and associated apolipoproteins may be bone fide targets for reducing the development of atherosclerotic lesions [30]. HDL-C plays a central role in reversal cholesterol transport, carrying cholesterol esters and triglycerides to the liver for removal via the bile secretion pathway [1]. In the present study, we found HDL-C levels were significantly raised by curcumin treatment, similar to the

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effect of lovastatin treatment. Increased plasma HDL-C levels are widely reported to be associated with decreased risk of atherosclerosis and cardiovascular disease [31]. Atherosclerotic lesion development could be inhibited via manipulation of reverse cholesterol transport components such as HDL-C, CETP and Apo A-I [32]. In plasma, curcumin treatment had no effect on Apo A-I, the major apolipoprotein component of HDL-C. However, we found the expression of Apo A-1 gene, which encodes the Apo A-I protein was significantly upregulated by curcumin and lovastatin in the liver. In contrast, plasma CETP activity that mediates the transfer of cholesteryl esters between HDL-C and LDL-C/VLDL was suppressed by curcumin and lovastatin treatment. The changes in the CETP activity are reported to be driven by the changes in plasma cholesterol, independent of LDL receptors [33]. Hence, it is unclear whether curcumin directly inhibits CETP activity. Nevertheless, elevated CETP levels are associated with the development of atherosclerosis [34] and we observed curcumin treatment effectively inhibited CETP activity.

4.4 Curcumin inhibits HMG-CoA activity via a transcriptional mechanism

%?>In the liver, HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis, and ACAT is the primary enzyme responsible for intracellular esterification of cholesterol. In the present study, we found that curcumin treatment inhibited hepatic HMG-CoA activity in vivo. We also observed that curcumin downregulated the HMGR gene, which encodes the HMG-CoA enzyme in liver. Furthermore, curcumin appeared to be a more potent inhibitor of HMGR transcription than lovastatin. Lovastatin acts as a reversible competitive inhibitor of HMG-CoA which is an enzyme substrate for HMG-CoA reductase in cholesterol biosynthesis [35]. Whether curcumin acts as a direct competitive inhibitor of HMG-CoA reductase similar to statins or primarily acts via an alternative mechanism remains to be determined. The present study does not rule out that the effects of curcumin on HMGR expression may be due to feedback inhibition as a result of suppressed HMG-CoA reductase activity. Nevertheless, we demonstrate that curcumin treatment was equally effective as lovastatin treatment for the inhibition of cholesterol biosynthesis in liver in vivo.

However, we observed no effect of curcumin or lovastatin on hepatic ACAT1 or ACAT2 expression, similarly ACAT activity was unchanged. ACAT1 is ubiquitously expressed, while ACAT2 is highly expressed in the liver. ACAT1 and ACAT2 encode acyl-coenzyme A acyltransferase enzymes responsible for the formation of cholesteryl esters. Evidence from ACAT1$^{-/-}$ and ACAT2$^{-/-}$ mice indicate that the loss of ACAT protein protects against atherosclerosis [26], due to lower cholesterol esterification. In the present study, we found that curcumin appears to lower cholesterol independent of changes in hepatic ACAT expression or ACAT activity. Our findings were consistent with previous reports that ACAT activity is unchanged by lovastatin treatment [36].

4.5 Curcumin modulates expression of lipid and cholesterol metabolism regulators

Curcumin suppressed accumulation of triglycerides and cholesterol in the liver; therefore, we examined whether curcumin altered both PPARα or LXRα nuclear receptors, which are established transcriptional regulators of lipid and cholesterol metabolism genes [37]. We found curcumin upregulated PPARα expression in a similar manner to lovastatin, which was consistent with the established role of PPARα in regulating fatty acid oxidation genes [37]. Hence, the upregulation of PPARα expression could partly explain the suppression of hepatic triglyceride accumulation we observed in curcumin- and lovastatin-treated mice. Furthermore, PPARα agonists directed at individual PPAR family members indicate activation of PPARα protects against macrophage foam cells and atherogenesis [38].

Curcumin treatment also increased LXRα transcription in liver. LXRα is another member of the nuclear factor super family. Oxysterols are natural ligands of LXRα, which initiate LXRα binding to promoter region of LXRα target genes. CYP7A1, which encodes cholesterol-7a-hydroxylase, is reported to be transcriptionally regulated by LXRα in mice [39]. Cholesterol-7a-hydroxylase plays a role in the conversion of cholesterol to bile acid, before removal to the intestine and excretion. Curcumin has been previously reported to increase cholesterol-7a-hydroxylase activity in high-fat diet fed rats [7]. LXRα also activates ATP-binding cassette, subfamily A1 (ABCA1) expression, which mediates reverse cholesterol transport from peripheral tissues back to the liver for removal via the bile synthesis pathway. However, some studies suggest that LXRα may also activate lipid synthesis under certain conditions, hence may contribute to hepatosis. Observations of LXRα activation of lipid synthesis tend to be short-term during the post-prandial state. Some studies involving potential LXRα agonists report increased lipid synthesis as a side effect [37]. However, in the present study increased LXRα was associated with suppressed hepatic triglyceride accumulation in curcumin- and lovastatin-treated mice.

There is growing interest in identifying novel PPARα and LXRα agonists, as multiple studies have demonstrated that PPARα or LXRα agonists may protect against atherogenesis [37, 38, 40]. However, whether curcumin is a natural ligand for either PPARα or LXRα remains to be established. The alteration in PPARα and LXRα appears to be consistent with the observed lower hepatic cholesterol and triglyceride accumulation in the curcumin-treated mice.

4.6 Curcumin suppresses systemic inflammation markers

Previous reports indicate that curcumin has widespread anti-inflammatory effects based on in vitro and pre-clinical

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studies [4]. We found curcumin reduced hsCRP, a major inflammatory biomarker which is produced during acute inflammation, and activates the complement pathway [41, 42]. CRP is often elevated alongside other cardiovascular risk factors; therefore, the role of CRP as a primary cause of atherogenesis remains controversial. Studies on transgene CRP expression in atherogenic models suggest that CRP accelerates atherosclerosis [43], while others suggest that CRP is neither pro-atherogenic nor pro-inflammatory [44]. No previous studies have reported that curcumin reduces systemic CRP levels in atherogenic susceptible animals. Nevertheless, our findings are consistent with the wider anti-inflammatory effects of curcumin reported by others in adipose tissue and hepatic tissue [45] as well as in chronic inflammatory disease models [4].

We also examined the in vivo effects of curcumin treatment on adipsin levels in plasma and Cfd expression in liver. Cfd is a factor in the alternative complement activation pathway, part of the innate immune system response, which directs pathogens to membrane attack complexes. Adipsin is identical to Cfd and is synthesized in adipocytes, as well as released from adipose tissue [46]. Here we found liver Cfd expression was dramatically suppressed by both curcumin and lovastatin treatment, although plasma adipsin levels were only lower in the lovastatin-treated mice. We previously reported that Cfd expression was upregulated in liver during the development of diet-induced obesity [47], consistent with earlier reports of increased expression of alternative complement pathway genes in atherogenic diet fed LDLR$^{-/-}$ mice [42]. Furthermore, LDLR$^{-/-}$ mice also lacking a complement factor gene, when fed an atherogenic diet, revealed that the activation of the alternative complement pathway plays an important role in atherogenesis [48]. Hence, we observed a previously unknown effect of curcumin on the immune complement pathway via transcriptional inhibition of Cfd in liver, which suggests the anti-atherosclerotic effects of curcumin, may be partly mediated via the suppression of complement pathway activation in the liver.

4.7 Limitations

In the present study, we used male LDLR$^{-/-}$ mice, while a well-established atherogenic model, no other studies examining the cholesterol-lowering effect have used LDLR$^{-/-}$ mice. The action of curcumin we observed herein is independent of the LDLR cholesterol uptake pathway, while curcumin in vitro is reported to alter the expression of LDLR in cultured liver cells [49]. Nevertheless, we examined several ways in which curcumin may act via hepatic cholesterol biosynthesis, as well as lipid and inflammatory regulators which have not been extensively reported. We recognize curcumin has other well-studied effects on antioxidant and lipid peroxide activity in high-fat or high-cholesterol fed animals which may also mediate the anti-atherogenic effect of curcumin reported herein [9, 11, 12, 15, 17, 18].

We acknowledge the differences in mRNA expression may not always translate into differences in protein level. However, for ACAT and HMGR gene expression, we also measured associated enzyme activity directly. While we focused on the mechanism of curcumin action in the liver, we cannot exclude the possibility that these are secondary changes due primarily to altered cholesterol absorption in the gastrointestinal tract.

It was not possible to examine whether curcumin had any dose-dependent effects on cholesterol metabolism. We choose 0.02% dietary curcumin based on extrapolation values of Generally Recognized As Safe (GRAS) by United States Food and Drug Administration which is also representative of dietary intake in South-Asian populations [4]. One of the earliest studies indicated no dose-dependent differences in the hypocholesterolemia effects of curcumin in rats fed at between 0.1 and 0.5% diet [5]. While higher doses of curcumin are reported to be well tolerated in mice [45], studies on the bioavailability of curcumin indicate poor absorption, high metabolism, inactivity of metabolic products or rapid clearance. At this point, improving the bioavailability of curcumin through nanotechnology holds great promise for a treatment of cancer or inflammatory bowel disease [50].

Although we could not discern whether the biological effects observed herein were due to curcumin or its metabolites, the observed effects of curcumin could partly be mediated by its metabolites as tissue levels of curcumin were reported to be very low [51]. Finally, based on the present data, 0.02% curcumin treatment was sufficient to protect against atherosclerosis in LDL receptor deficient mice.

4.8 Future directions

It is well established that curcumin lowers plasma cholesterol levels and hence strongly suggests that curcumin may protect against atherosclerosis in high-cholesterol fed animals [6–13]. We show here that curcumin also modulates hepatic gene expression and inhibits cholesterol biosynthesis. While evidence from animal studies is accumulating, there is a paucity of studies on the effects of curcumin on plasma cholesterol management in humans [3, 4]. While we analyzed the effects of curcumin and lovastatin alone, it may be worthwhile to investigate whether a combination of curcumin with existing lipid-lowering agents is more effective for cholesterol management.

4.9 Concluding remarks

Curcumin treatment was able to effectively suppress atherosclerotic lesion development in LDLR$^{-/-}$ mice, which are

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highly susceptible to atherosclerosis when fed a high cholesterol diet. The anti-atherogenic effects of curcumin appear to be mediated via action in the liver. Curcumin transcriptionally inhibits HMG-CoA activity, independent of ACAT activity. We also observed upregulation of the nuclear transcriptional factors PPARα and LXRα in liver by curcumin treatment in vivo. Furthermore, we showed curcumin also downregulated the complement activation pathway gene (Cfd) in liver, independent of systemic adipsin levels. Taken together, these findings indicate that curcumin protects against atherosclerosis via multiple mechanisms including altered lipid, cholesterol and immune gene expression.

In conclusion, curcumin was equally effective as lovastatin for lowering cholesterol and protecting against atherosclerosis. Hence, dietary curcumin may ameliorate the need for pharmacological control of cholesterol levels by statins, but this would need to be established in long-term clinical trials.

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