Full Length Research Paper

Celastrol attenuates atherosclerosis in Apolipoprotein E (apoE) knockout mice fed an atherogenic diet

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The study is aimed to investigate the effects of celastrol on the atherogenesis in apolipoprotein E knockout (apoE$^{-/-}$) mice fed an atherogenic diet. All mice were fed a high-fat and high-cholesterol diet for 8 weeks. In the last 4 weeks, apoE$^{-/-}$ mice were randomly grouped to receive treatment with either celastrol (2 mg/kg/day in DMSO, i.p.) or vehicle DMSO (n=6 per group). Plasma lipids were determined enzymatically. The area of atherosclerotic lesions was measured by hematoxylin and eosin staining. The expression of macrophage migration inhibitory factor (MIF), matrix metalloproteinase-9 (MMP-9), CD40 ligand (CD40L) and C-reactive protein (CRP) were assayed by immunohistochemistry. Celastrol inhibited the development of atherosclerotic lesions in apoE$^{-/-}$ mice. The expressions of MIF, MMP-9, CD40L and CRP in the artery wall were significantly reduced by celastrol. Meanwhile, the lipid profile in the mice was not improved. In conclusion, our study demonstrates that celastrol inhibits atherogenesis in celastrol-treated apoE$^{-/-}$ mice fed an atherogenic diet by inhibiting inflammation in the arterial wall without improving the lipid profile.

Key words: Celastrol, atherosclerosis, CD40 ligand, macrophage migration inhibitory factor, matrix metalloproteinase-9.

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease (Lusis, 2000). A complex endothelial dysfunction induced by modified low-density lipoproteins, free radicals, infectious microorganisms, shear stress, hypertension, toxins after smoking or combinations of these and other factors leads to a compensatory inflammatory response (Ross,1999). The vascular inflammatory reaction involves complex interactions between inflammatory cells and vascular cells, including signaling via NF- B (Collins, 1993). Blocking of inflammatory mediators can decrease the atherosclerotic lesion size (Libby, 2002; Ross, 1999).

Platelets play a main part in inflammation as well (Gawaz et al., 2005; Weber, 2005). Platelet activation has recently been recognized as an important factor in inflammatory processes. The platelet surface molecules P-selectin and GPIb can bind to P-selectin glycoprotein ligand-1 (PSGL-1) and Mac-1 ($_M$ 2, CD11b/CD18) on leukocytes respectively (Ehlers et al., 2003; von Hundel-

Hundelshausen and Weber, 2007) which triggers leukocyte arrest and induces transendothelial migration of leukocytes. It is commonly assumed that platelets function as immune cells bridging inflammation and cardiovascular disease (von Hundelshausen and Weber, 2007). Therefore, platelet activation is a common feature in inflammatory diseases and occurs not only in cardiovascular pathologies, such as atherosclerosis, restenosis, thrombosis, and coagulation, but also in sepsis, inflammatory bowel disease, or arthritis (May et al., 2008).

Celastrol has been proved to be anti-oxidant, anti-inflammatory, anti-cancer, and immunosuppressive agent (Corson and Crews, 2007). The therapeutic effects of celastrol have been observed in animal models of lupus (Li et al., 2005), collagen-induced or rheumatoid arthritis (Li et al., 1997; Tao et al., 2002), and Alzheimer's disease (Allison et al., 2001). Intraperitoneal injection of celastrol (1 to 3 mg/kg/day for 31 days), has anti-proliferative effects against prostate cancer in nude mice (Yang et al., 2006). Our recent study has demonstrated that celastrol inhibits platelet activation, adhesion to fibrinogen and aggregation *in vitro*, as well as fibrinogen binding of platelets in celastrol-treated mice, which may contribute

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to explain celastrol's anti- inflammatory and immunosuppres-sive effects (Hu et al., 2009). Furthermore, our previous data indicate that celastrol may also have anti-thrombotic properties. Nevertheless, the effect of celastrol on atherosclerosis is still unclear. In this study, we investigated the effects of celastrol on the formation and the plaque stability of atherosclerosis in the apolipoprotein E knockout (apo E $^{-/-}$) mice. The expression of macrophage migration inhibitory factor (MIF), matrix metalloproteinase (MMP- 9), CD40 ligand (CD40L) and C-reactive protein (CRP) in atherosclerotic lesions were assayed by immunohistochemistry as well.

MATERIALS AND METHODS

Dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA) was purchased from Sigma (St. Louis, USA). Celastrol was purchased from Calbiochem (Merck, Darmstadt, Germany) and was dissolved in DMSO. The rabbit anti-CRP, anti-MIF, anti- MMP-9 polyclonal antibody were obtained from Santa Cruz (USA).

Animals and diets

Twelve male 8-week-old apoE-/- mice were purchased from the Centre for Experimental Animals, Peking University Health Science Center (China). Six 8-week-old male C57BL/6J mice (obtained from the Laboratory Animal Center of Third Military Medical University) were used as wild-type (WT) control animals. All mice were fed a high-fat/high-cholesterol diet (HFC, comprising, wt/wt, 20% protein, 50% carbohydrate, 21% fat, and 0.21% cholesterol) for 8 weeks. In the last 4 weeks, apoE $^{-/-}$ mice were randomly grouped to receive treatment with either celastrol (2 mg/kg/day in DMSO, i.p.) or vehicle DMSO (n=6 per group). The C57BL/6J mice were treated with DMSO as well. At the end of experiments, mice were sacrificed and blood samples collected from the abdominal aorta. The blood was mixed with the anti-coagulant Na$_2$EDTA (1.5 g/L blood). Plasma was obtained by centrifuging the blood at 800 g at 4°C for 10 min and stored at -80°C until use. The aorta was collected for the lesion analyses. All animal experiments were done in accordance with the Institutional Animal Ethics Committee and the Third Military Medical University Animal Care guidelines for use of experimental animals.

Analyses of plasma lipids and atherosclerotic lesions

Plasma concentrations of cholesterol and triglyceride were determined enzymatically with Sterozyme 545 and Determiner LTG assay kits (Kyowa Medics, Tokyo, Japan), respectively, in automatic biochemistry analyzer (Olympus Au2700).

Each aorta from the arch to the femoral bifurcation was cleaned free of connective tissue and fat. From the aortic root, a 600 m long vessel was obtained, and frozen section was made by a 10 m interval, stained with hematoxylin and eosin (H&E). The atherosclerotic lesion area was then calculated in each section.

Expression of MIF, MMP-9, CD40L and CRP in atherosclerotic lesions

Aortic root tissues were imbedded in OCT (Optimal Cutting Temperature) compound for cryostat sectioning. Five 5- m-thick cryostat sections from each sample were selected by 100 m interval, and used for immunohistochemistry. Briefly, slides were air dried for 30 min, and fixed with acetone for 10 min. Goat polyclonal anti-MMP- 9, anti-CD40L, and anti- CRP antibodies and rabbit polyclonal anti-MIF antibody (Santa Cruz, USA) were used to determine the expression of each protein. Tissue sections were incubated with each antibody (1:200 dilution) for 2 h at room temperature. After rinsing in PBS, the sections were incubated with biotinylated secondary antibody for 30 min. The slides were then incubated with avidin-biotin complex for 30 min. The sections were again rinsed in PBS and incubated with diaminobenzidine for 10 min in the dark. After chromogen development, slides were washed in distilled water and counterstained with H&E.

Brownish yellow granular or linear deposits in the cells or matrix were interpreted as positive areas. Semi- quantitative evaluation was performed by Image-Pro Plus 5.0. The integrated optical density (IOD) of the positively stained area was measured at 400X magnification in 5 random fields for every slide, and 3 slides were assessed for each protein in one mouse. The data were expressed as IOD per group.

Statistical analysis

Data are expressed as means ± SD. Comparisons among groups were performed by student's t test using SPSS13.0 software. Differences were considered significant at a value of $p < 0.05$ for all tests.

RESULTS

Effects of celastrol on body weight and plasma lipid profile in mice with HFC –diet

To determine whether celastrol treatment affects body weight and plasma lipids in apoE $^{-/-}$ or WT mice with HFC-diet, we measured these parameters and found that apoE-/- mice fed HFC-diet plus celastrol treatment for 4 weeks showed significantly decreased body weight when compared with vehicle control group. Though the celastrol treatment group has lower total cholesterol (TC) and HDL-c levels than that in DMSO group, but the atherogenic index, (TC - HDL-c)/HDL-c, in celastrol treatment group was 40.6, while it was 26.7 in vehicle control group (Table 1).

Atherosclerotic lesions in the mouse aorta

To examine the impact of celastrol on atherogenesis in apoE $^{-/-}$ mice, atherosclerotic lesions were evaluated by aortic root section analyses after 4 weeks on HFC diet in the absence or presence of celastrol. Compared with the control vehicle-treated group, the area of atherosclerotic lesion in the aortic root of apoE $^{-/-}$ mice reduced by 41.1% in celastrol group (p<0.05; Table 2). Representative H&E-stained aortic sections are shown in Figure 1.

Expression of MIF, MMP-9, CD40L and CRP Protein in the apoE $^{-/-}$ mouse aorta

One potential mechanism underlying reduced atherosclerosis in apoE $^{-/-}$ mice treated with celastrol is

Table 1. Effects of celastrol on body weight and lipid profile in mice with HFC-diet (n = 6, means±SD).

Parameter	WT/DMSO	apoE$^{-/-}$/DMSO	apoE$^{-/-}$/CeT
B.W. (g, 8 w)	19.1±0.6	19.5±0.6	19.9±0.9
B.W. (g,12 w)	23.3±0.3	27.6±0.9	27.1±1.2
B.W. (g,16 w)	24.3±0.4	28.8±1.4	24.4±1.5*
TC (mmol/l)	3.40±1.05	19.38±3.06#	14.97±2.08*
HDL-c (mmol/l)	1.04±0.26	0.70±0.23#	0.36±0.14*
TG (mmol/l)	0.33±0.08	0.67±0.15#	0.65±0.21

B.W., Body weight; TC, total cholesterol; HDL-c, high density lipoprotein-cholesterol; TG, triglyceride; WT, wild-type mice (C57BL/6J); DMSO, dimethyl sulfoxide; CeT, celastrol. *p 0.05 (*vs.* apoE$^{-/-}$/DMSO group), # p 0.05 (*vs.* WT/DMSO group).

Table 2. Aortic root section analyses of atherosclerotic lesion area in apoE$^{-/-}$ mice (n=6, means±SD).

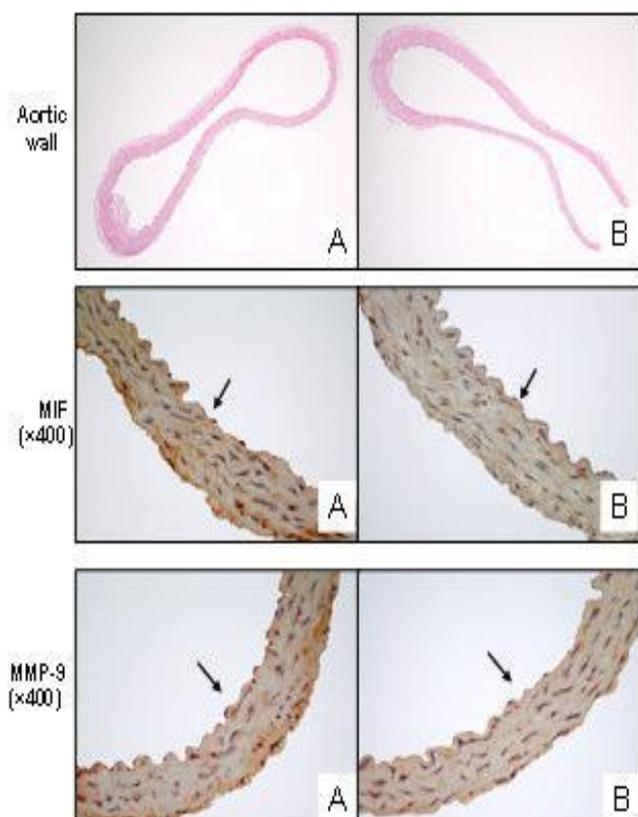| Group	Lesion area (m^2)	Vessel wall area (m^2)	Lesion / vessel wall ratio
Vehicle group	8403±2535	103720±21467	0.082±0.021
Celastrol group	4947±1277*	100134±14617	0.050±0.017*

*p 0.05 *vs.* vehicle group (DMSO).

Figure 1. Representative aortic root sections staining with H&E, and the expression of MIF, MMP-9 with immunohistochemistry staining in apoE$^{-/-}$ mice from vehicle-treated HFC-diet group (A) and celastrol-treated HFC-diet group (B). Arrows indicate endothelium.

Table 3. The expression of MIF, MMP-9, CD40L and CRP in the aortic wall of apoE$^{-/-}$ mice ($\times10^{-4}$, n=6, means±SD).

IOD	apoE$^{-/-}$/DMSO	apoE$^{-/-}$/CeT
MIF	227±39	114±16**
MMP-9	263±80	54±20**
CD40L	250±70	152±14**
CRP	256±26	152±52**

IOD, Integrated optical density; DMSO, dimethyl sulfoxide; CeT, celastrol; MIF, macrophage migration inhibitory factor; MMP-9, matrix metalloproteinase; CD40L, CD40 ligand; CRP, C-reactive protein. **p 0.01 (vs. apoE$^{-/-}$/DMSO group).

through inhibition of inflammation in the vascular wall. To examine this possibility, we analyzed expression of MIF, MMP- 9, CD40L and CRP protein in the artery lesions using antibody against each protein. After immunostaining of the aortic root sections, semi-quantitative analyses was performed, and the results showed that the MIF, MMP-9, CD40L and CRP expression in celastrol group were substantially lower than that in the vehicle group (p<0.01) (Table 3). Representative images of immunohistochemis-try are shown in Figure 2.

DISCUSSION

The triptergium vine has been used for several centuries in China for the treatment of arthritis and other inflammatory diseases. It has been demonstrated that the
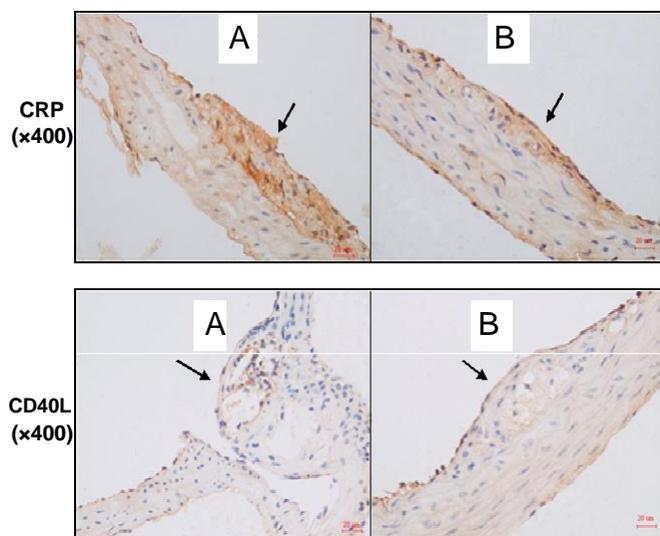
Figure 2. The expression of CRP and CD40L with immunohistochemistry staining in the aortic root sections of apoE$^{-/-}$ mice from vehicle-treated HFC-diet group (A) and celastrol-treated HFC-diet group (B). Arrows indicate endothelium.

tripterygium vine which contains 70 active ingredients has multiple pharmacological effects such as anti-neoplasmic and anti-proliferative actions as well as effects of immune suppression (Chen et al., 2005; Corson and Crews, 2007). The postulated therapeutical mechanisms of celastrol include a potentiation of TNF- induced apoptosis, inhibition of tumor cell invasion through suppression of the NF- B pathway (Sethi et al., 2007), proteasome inhibition (Yang et al., 2006), inhibition of cyclooxygenase-2 and prostaglandin E$_2$ (Tao et al., 1998), as well as activation of heat shock and antioxidant response (Trott et al., 2008; Westerheide et al., 2004). However, many effects of this medicine, especially on atherosclerosis, remain unclear.

The present study demonstrates that celastrol inhibits atherogenesis through its anti-inflammatory effects, such as inhibition of the expression of MIF, MMP-9, CD40L and CRP in atherosclerotic lesions. Meanwhile, the lipid profile was not improved by celastrol treatment. MIF is a pleiotropic cytokine that regulates a broad spectrum of inflammatory reactions including atherosclerosis (Hoi et al., 2007). Lin et al. demonstrated that expression of MIF by endothelial cells and vascular smooth muscle cells may play a role in macrophage adhesion, transendothelial migration, accumulation, and importantly, transformation into foam cells (Lin et al., 2000). Chen et al. analyzed murine MIF proximal promoter region and found two consensus NF- B binding sites (Chen et al., 2009).As celastrol can inhibit the invasion of tumor cells through suppression of the NF- B pathway (Sethi et al., 2007), we speculate that celastrol reduces MIF expression in atherosclerotic lesions at least in part by inhibiting NF- B pathway.

The expression of MMPs, including MMP-9, play an important role in the rupture of atherosclerotic plaque. Expression of an autoactivating form of MMP-9 in macrophages *in vitro* greatly enhances elastin degradation and induces significant plaque rupture when overexpressed by macrophages in advanced atherosclerotic lesions of apoE$^{-/-}$ mice *in vivo* (Gough et al., 2006). The role of CD40/CD40 ligand (CD40L) in atherothrombosis is now widely accepted. CD40L and sCD40L are molecules with a dual prothrombotic and proinflammatory role (Antoniades et al., 2009). In conclusion, our study demonstrates that celastrol inhibits atherogenesis in apoE knockout mice fed an atherogenic diet by inhibiting inflammation in the arterial wall.

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