Atorvastatin inhibits collar-induced intimal thickening of rat carotid artery: Effect on C-type natriuretic peptide expression

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Abstract. The present study was performed to elucidate the mechanism underlying the anti-atherogenic action of atorvastatin (ATV). We investigated the effect of ATV on intimal thickening of the rat carotid artery induced by collar placement and further examined the effect of ATV on the expression of C-type natriuretic peptide (CNP), cGMP-dependent protein kinase (PKG) Iα and PKG Iβ in carotid arteries. The expression of CNP was examined by enzyme-linked immunosorbent assay (ELISA) and quantitative real-time RT-PCR. Western blotting was used to determine the expression of PKG Iα and PKG Iβ. After 14 days, the collar placement induced a marked neointima formation and a reduction in CNP and PKG Iα expression. These effects were significantly reversed by ATV treatment. However, no obvious changes in PKG Iβ expression were observed throughout the study. In conclusion, the present data suggest that elevation of CNP represents an additional mechanism by which ATV treatment may prevent the development and progression of atherosclerosis.

Introduction

Atherosclerosis, the major cause of cardiovascular disease, is a multifaceted, progressive, inflammatory disease in which the formation and build-up of atherosclerotic plaques cause hardening and narrowing of major arteries. Atherosclerosis may lead to several important adverse vascular events including coronary artery disease (CAD), stroke and peripheral arterial disease, responsible for most of the cardiovascular morbidity and mortality worldwide (1-3). Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have emerged as effective agents in lowering elevated levels of low-density lipoprotein-cholesterol (LDL-C), one of the principal risk factors for atherosclerosis and its pursuant manifestations (4). Reductions in total LDL-C achieved by statin treatment have been shown to translate into reductions in the risk of cardiovascular morbidity and mortality in both primary and secondary prevention settings (5-8). However, there is increasing evidence for additional benefits of statins that cannot be fully explained by their lipid-lowering effect (9,10). Several clinical trials demonstrated anti-inflammatory and immunomodulatory effects of treatment with statins that would represent the likely explanations for further benefits attributable to this class of drugs (11-13). Nonetheless, the mechanisms underlying the anti-inflammatory effect of statin treatment have not yet been fully elucidated.

C-type natriuretic peptide (CNP), a 22-amino acid peptide, belongs to a family of structurally related cardiovascular hormones also including atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP). ANP and BNP are synthesized in cardiomyocytes and released into the circulation in significant amounts, thereby contributing to the regulation of cardiovascular homeostasis. In contrast, CNP is secreted by vascular endothelial cells and acts locally as a regulator of vascular tone and growth through intracellular accumulation of cyclic guanosine monophosphate (cGMP) (14,15). Furthermore, CNP appears to have both anti-inflammatory and anti-mitogenic properties, suggesting that it may also be protective against the development of atherosclerotic lesions (16,17). In support of this, CNP has been reported to inhibit the intimal thickening in a number of experimental models of atherosclerosis, and expression of CNP and its receptors NP receptor (NPR)-B and -C in human coronary arteries are inversely correlated with severity of atherosclerotic lesions (18-21). Additionally, CNP also exerts anti-migratory and anti-proliferative effects on vascular smooth muscle cells (VSMCs) *in vitro* (15).

Based on these previous studies on statins and CNP, we raised the hypothesis that statins may protect against the development of atherosclerosis, at least partially through the elevation of CNP expression. To test our hypothesis, we examined the effects of atorvastatin (ATV) treatment on intimal thickening and CNP expression in collar-induced atherosclerotic rats.

Materials and methods

Animal model of atherosclerosis. Male adult rats weighting from 220 to 250 g were purchased from the Experimental

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Animal Centre of China Medical University and fed a regulatory diet (zero cholesterol) 1 week before surgery. Rats were anesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg). Subsequently, a midline neck incision was made to surgically expose the right carotid artery, and a non-occlusive silastic collar (length 10 mm, inner diameter 1.5 mm) was positioned around the right carotid artery and held in place with a nylon sleeve. The carotid arteries were then returned to their original position and the entry wound was sutured. After recovery from the anesthesia, the animals received their respective treatment for 2 weeks. The rats in the sham group underwent the same procedure except the collar placement. All animal study protocols were approved by the Ethics Committee for Animal Experiments of China Medical University, and the animals received human care in compliance with the Principles of Laboratory Animal Care.

Experimental design. After surgery, rats were divided into two groups receiving ATV at a dose of 10 mg/kg/day or vehicle (5% glucose solution), respectively. The dosage of ATV administered in this study was based on previous studies (22-24). Rats were assigned randomly to each of the treatment groups and received ATV or vehicle for 14 consecutive days by oral gavage. The sham-operated rats received a regulatory diet only. During the study, animals were provided *ad libitum* with a standard diet and water.

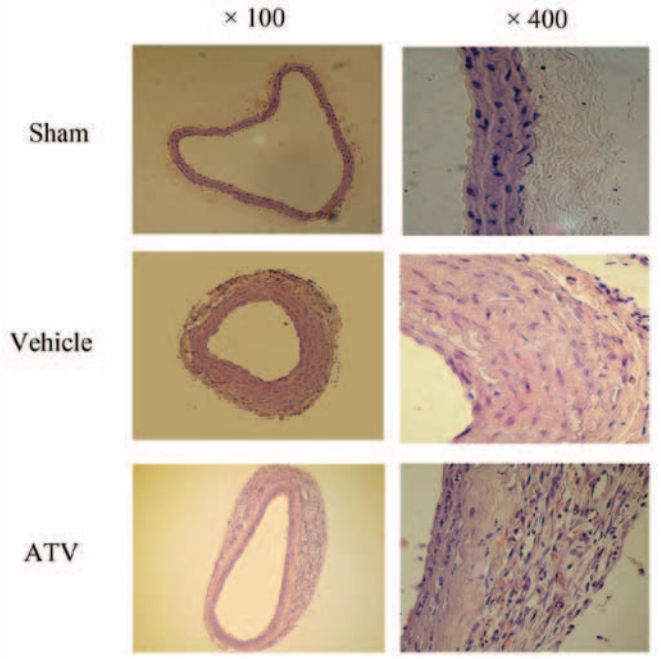

Figure 1. Photomicrographs of paraffin transverse sections of carotid arteries after staining with haematoxylin and eosin (H&E).

Tissue harvesting and morphometry. At the end of the study, all rats were sacrificed with an overdose of phenobarbital and two segments were cut from the collared and sham-operated artery, one for western blot analysis, the other for morphometry. The former was immediately frozen at -80˚C until needed. The latter was immediately placed in formalin fixative solution (4%) for 24 h, dehydrated in a graded series of isopropyl alcohol (60-100%) followed by toluol before being embedded in paraffin. Transverse 5-μm tissue sections were cut and routinely stained with hematoxylin and eosin (H&E). The cross-sectional area of the intima and media in each artery and the ratio of these values were calculated (intima/media area ratio, IMR).

Enzyme-linked immunosorbent assay (ELISA). CNP expression in artery segments was assayed using an ELISA kit (EIAab Science Co., Ltd, Wuhan, China). In brief, total proteins were extracted from artery segments and the protein concentrations were determined by the bicinchoninic acid (BCA) assay. Then, CNP concentrations by ELISA were carried out according to the manufacturer's instructions.

Quantitative real-time RT-PCR. Total-RNA was isolated from frozen artery segments using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration and purity of the RNA in each sample were determined using a spectrophotometer at 260 and 280 nm. cDNA was synthesized from 1 μg of total-RNA using a PrimeScript RT reagent kit (Takara, Dalian, China). Quantitative real-time RT-PCR was performed using SYBR-Green (Takara Biotechnology) on an Exicycler™ 96 real-time quantitative thermal block (Bioneer, Daejeon, Korea). The PCR primer sequences were designed according to the rat CNP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences reported in GenBank and were chemically synthesized: CNP, forward, 5'-CACCATGCACCTCTCCCAGC-3' and reverse, 5'-ATGGAGCCGATCCGGTCCAG-3'; GAPDH, forward, 5'-GCAAGTTCAACGGCACA-3' and reverse, 5'-CATTTGATGTTAGCGGGAT-3'. The specificity of the amplified products was analyzed through dissociation curves generated by the equipment yielding single peaks. GAPDH was used as an internal control to normalize samples. PCR reactions of each sample were conducted in triplicate. Data were analyzed through the comparative threshold cycle (CT) method.

Western blot analysis. The frozen artery segments were lysed in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) and the protein concentrations were determined by the BCA assay. Equal amounts of protein were separated by SDS-PAGE and then electrotransferred to PVDF membranes (Millipore, Billerica, MA). The blotted membranes were blocked with 5% skim milk at 4˚C overnight, and then incubated with goat anti-cGMP-dependent protein kinase (PKG) Iα or anti-PKG Iβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1000) at room temperature for 2 h. After incubation with the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, visualization was performed by an enhanced chemiluminescence kit. Immunoblotting with the anti-actin antibody was used as an internal control to confirm equivalent protein loading.

Statistical analyses. Numerical data are presented as mean ± SD and were analyzed by the Student's t-test using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered to denote statistical significance.

Table I. Effect of atorvastatin (ATV) treatment on neointimal information in carotid arteries.

Group	Sham	Vehicle	ATV
Medial area (mm^2)	0.130±0.008	0.066±0.002[a]	0.060±0.003[a]
Intimal area (mm^2)	undefined[c]	0.009±0.001	0.004±0.001[b]
I/M ratio	undefined	0.13±0.05	0.06±0.01[b]

[a]Significantly different from the sham group (P<0.01); [b]significantly different from the vehicle group (P<0.01). [c]Undefined, due to the lack of intimal growth. Values are expressed as the mean ± SD from 8-10 experiments.

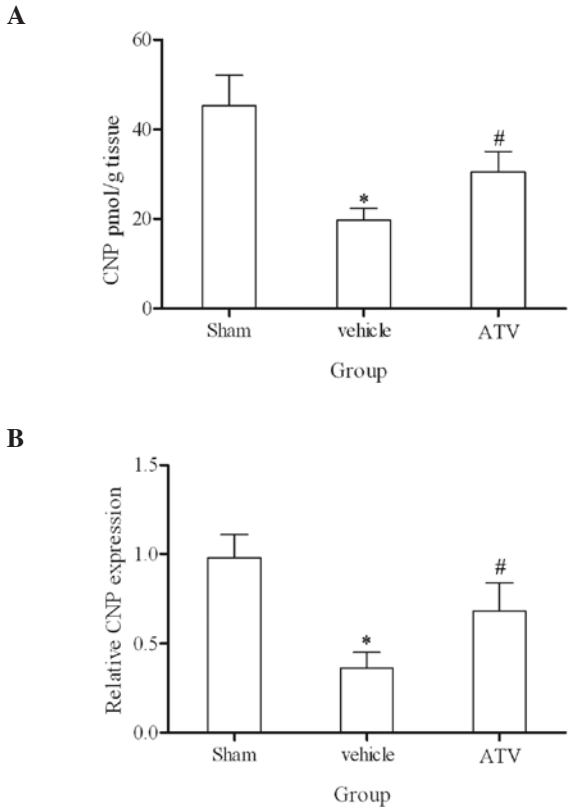

Figure 2. (A) Expression of CNP in carotid arteries as determined by ELISA assays and (B) quantitative real-time RT-PCR. *P<0.05 v.s. sham group; #P<0.05 v.s. vehicle group.

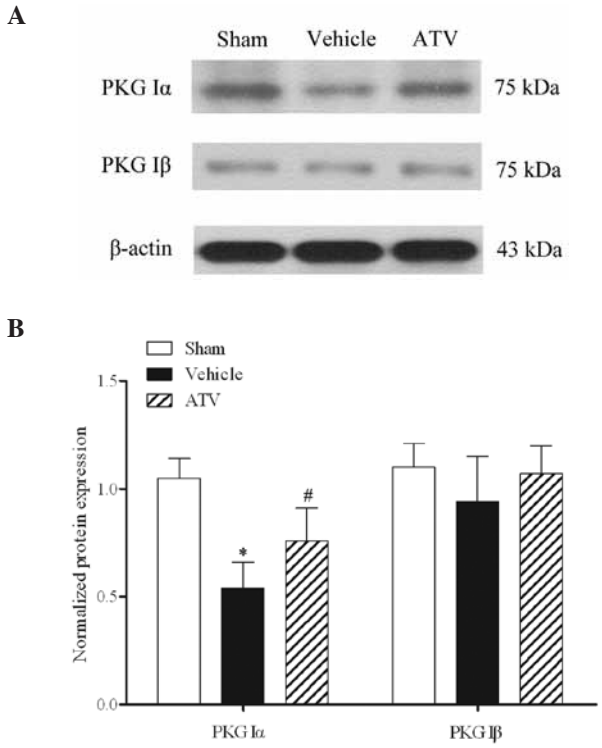

Figure 3. Protein levels of PKG Iα and PKG Iβ in carotid arteries. (A) Representative western blotting of three independent and reproducible experiments. (B) Quantitative data were expressed as the intensity ratio of the target genes to GAPDH.

Results

Effect of ATV on neointima formation induced by perivascular collars. In the present study we investigated the effect of ATV administration on the development of an atheroma-like neointima induced by application of a peri-arterial collar in rats. As shown in Fig. 1 and Table I, the medial area in both the vehicle- and ATV-collared arteries was significantly reduced when compared to the sham segment. In collared arteries, ATV treatment did not affect the medial changes but it significantly suppressed the neointimal growth showing a reduction in both intimal area and IMR when compared to vehicle treatment. The sham segment remained unaffected (Table I). These observations suggest that administration of ATV was effective in preventing the development of a neointima in the atherosclerotic rat model.

Effect of ATV on CNP expression in carotid arteries. To further explore the possible mechanisms by which ATV prevents the formation of a neointima, we examined the concentration and mRNA level of CNP in carotid arteries using ELISA and quantitative real-time RT-PCR, respectively. As shown in Fig. 2A, in comparison to the arteries in the sham group, the concentrations of CNP were markedly reduced in the vehicle group (P<0.05). However, ATV treatment significantly attenuated this reduction (P<0.05). Meanwhile, changes observed by quantitative real-time RT-PCR study were in accordance with the findings in the ELISA assays (Fig. 2B). These findings suggest that ATV suppressed the neointimal growth possibly by elevating CNP expression.

Effect of ATV on the expression of PKG Iα and PKG Iβ in carotid arteries. CNP has long been known as a potent

stimulator of cGMP (25). To provide more evidence for the elevation of CNP in carotid arteries, we further examined the protein expression of two PKGs (PKG Iα and Iβ), the serine/threonine-specific protein kinase that is activated by cGMP. Compared with the sham group, there was a marked decrease in the PKG Iα protein level in the vehicle group. This reduction was significantly reversed by ATV treatment ($P<0.05$, Fig. 3A and B). However, no obvious changes in PKG Iβ expression were noted among the three groups (Fig. 3A and B). These results not only confirmed the elevation of CNP by ATV treatment, but also indicate that the inhibition of neointimal growth by ATV may be associated with the activation of PKG Iα.

Discussion

The present study demonstrated, for the first time, that ATV treatment in the peri-arterial collar model of atherosclerosis in rats prevented the formation of a neointima, usually noted 7 days after the placement of the peri-arterial collar alone. In addition, ATV treatment increased the expression of CNP and PKG Iα in carotid arteries. Therefore, ATV may have a protective role in atherosclerosis and this role may prove beneficial in reducing the remodeling associated with the early stages of atherosclerosis.

Placement of a pericarotid or perifemoral collar has been shown to induce intimal thickening in rabbits, rats and mice (26-28). Previous studies have demonstrated that collar placement causes polymorphonuclear leukocyte infiltration and endothelial injury in the initial step by inflammatory responses, followed by the migration of medial vascular smooth muscle cells to the intima and the proliferation of migrated vascular smooth muscle cells with deposition of extracellular matrix in the neointima. These steps are comparable to those observed in the development of human atherosclerosis (29). Consequently, intimal thickening induced by collar placement may be an adequate model to facilitate understanding of atherosclerosis. In this study, intimal thickening of the rat carotid artery was induced by collar placement. Consistent with previous results, a marked neointima formation was observed in the carotid artery of the vehicle group two weeks after the collar treatment, whereas this neointima was significantly inhibited by the ATV treatment. These findings not only confirmed the successful induction of the atherosclerotic rat model, but also suggest that ATV treatment is capable of preventing the development of a neointima.

The protective effects of ATV and CNP on atherosclerosis have been separately reported in different models. For example, Raval *et al* recently showed that ATV combined with celecoxib, a cyclooxygenase (COX-2) inhibitor, reduced the extent of atherosclerosis and inflammatory/cell adhesion molecule levels in the apo $E^{-/-}$ mouse model (30). In addition, Gaspari *et al* have demonstrated that local infusion of CNP results in preservation of endothelial function and prevention of neointimal thickening in collar-induced atherosclerotic rabbits (31). Nevertheless, a direct association between ATV and CNP has never been described. In the present study, we found that ATV treatment inhibits the neointima formation in an atherosclerotic rat model, which was accompanied by increases in CNP expression, suggesting that ATV may exert its protective effect by upregulating CNP. Furthermore, previous investigations have clearly demonstrated that the anti-inflammatory mechanisms of statins may involve upregulation of transforming growth factor β (TGF-β) (32,33). In addition, the expression of CNP is reported to be influenced by several physiological and pathological mediators relevant to the cardiovascular system including upregulation by TGF-β and downregulation by oxidized LDL (15). Based on these findings, it therefore seems reasonable to propose that activation of the TGF-β signaling pathway may represent one underlying mechanism for the protective effect on atherosclerosis by upregulating the expression of CNP. However, the precise mechanisms by which ATV increases the CNP expression awaits further elucidation.

In conclusion, ATV treatment inhibited the development of a neointima in an atherosclerotic rat model, accompanied by the increase in CNP expression in carotid arteries. Thus, the present data suggest that elevation of CNP represents an additional mechanism by which ATV treatment may prevent the development and progression of atherosclerosis.

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