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Statins exert differential effects on angiotensin II-induced atherosclerosis, but no benefit for abdominal aortic aneurysms

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ABSTRACT

Objective: Statins reduce atherosclerosis, but it is controversial whether they suppress abdominal aortic aneurysm (AAA) expansion. We hypothesized that statins (rosuvastatin and atorvastatin) would attenuate angiotensin II (AngII)-induced atherosclerosis and AAA.
Methods and results: Sixty apoE−/− male mice fed a normal diet were administered with either rosuvastatin (10 mg/kg/day) or atorvastatin (20 mg/kg/day) through drinking water for 1 week prior to initiating 28-day AngII infusion (1000 ng/kg/min). Statins administration led to therapeutic serum concentrations of drugs. Administration of either rosuvastatin or atorvastatin exerted no significant effect on AngII-induced expansion of suprarenal diameter or area. However, atorvastatin significantly reduced AngII-augmented atherosclerotic lesion areas in intimas of both aortic arches and cross-sections of aortic roots ($P < 0.001$). Atherosclerosis was attenuated independent of reductions in serum total cholesterol concentrations. Although serum MCP-1 and MIF concentrations were not changed by either statins, atorvastatin administration increased PPAR-α and -γ mRNA abundances and decreased NF-κB p50, p65, MCP-1 and TNF-α mRNA abundances in atherosclerotic lesions.
Conclusions: This study demonstrated both statins failed to suppress AngII-induced AAA. In contrast, atorvastatin reduced AngII-induced atherosclerosis associated with no change in serum inflammatory markers but a shift to upregulation of anti-inflammatory status in lesions.

1. Introduction

Although abdominal aortic aneurysm (AAA) and atherosclerosis are chronic inflammatory vascular diseases, the pathogenetic mechanisms are disparate. However, the renin-angiotensin system (RAS) is involved in both the development of AAA and the pathogenesis of atherosclerosis by stimulating a series of coordinated cellular and molecular events [1,2]. Angiotensin II (AngII) contributes to acceleration of atherosclerosis and induction of aneurysm by promoting complex changes of arteries that are independent of blood pressure [3]. Subcutaneous infusion of AngII into hypercholesterolemic mice both induces AAA formation and augments atherosclerosis [1,2]. In contrast, blockade of AngII suppresses development of AAAs associated with decreased macrophage accumulation and matrix metalloproteases (MMPs) expression [4].

Administration of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) inhibitor (statin) is a highly validated therapeutic approach for patients with coronary heart disease [5]. Reductions of cardiovascular morbidity and mortality with statin therapy have been attributed to lowering of plasma cholesterol concentrations and other non-lipid-based mechanisms, such as improved endothelial function and anti-inflammatory effects [6]. Rosuvastatin and atorvastatin are two of the most widely prescribed statins that profoundly reduce plasma concentrations of total cholesterol and low-density lipoprotein cholesterol (LDL-C). It has been suggested that administration of rosuvastatin can reach the recommended lipid targets more expeditiously than atorvastatin at equivalent doses [7]. Several anti-atherogenic mechanisms have been proposed for atorvastatin, such as reducing inflammatory chemokines expression by inhibition of NF-κB signaling and activation of inducible NO synthase expression [8,9], decreasing

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MMP-9 content, and increasing collagen and fibrous cap thickness in atherosclerotic lesions [10]. Recent studies have shown the anti-atherogenic effects of rosuvastatin administration associated with anti-inflammatory and anti-atherothrombotic properties [11].

However, the effects of statins on AAAs have not been fully defined. Most reports focus on the beneficial effects of statins for AAA therapy on protease and inflammatory markers in plasma or aneurysmal wall [12,13]. It is controversial whether statins can prevent the expansion and rupture of human AAAs [14]. Atorvastatin has been demonstrated to suppress the development of AAAs in elastase-induced rats through inhibition of macrophage migration [15]. No studies have defined the effects of rosuvastatin on AAA.

We hypothesized that both statins would suppress development of AngII-induced AAAs and atherosclerosis. Rosuvastatin and atorvastatin, as the representatives of hydrophilic and lipophilic statins, respectively, were administered into AngII-infused apoE−/− male mice. These drugs were administered in drinking water at concentrations that achieved serum concentrations that were sufficient to inhibit mouse HMG-CoA reductase. AngII (1000 ng/kg/min) was infused subcutaneously to promote development of AAAs and augment atherosclerotic lesions [1]. Serum inflammatory markers and associated transcription factors in lesions were analyzed to provide a basis for potential mechanisms.

2. Materials and methods

2.1. Animals

ApoE-deficient (apoE−/−) mice on C57BL/6 background ($n = 60$) were originally purchased from the Beijing Vital River Laboratory Animal Technology Corporation and bred in-house. Mice were maintained under specific pathogen-free (SPF) conditions and fed normal laboratory diet. All studies were performed with the approval of Zhejiang University Institutional Animal Care and Use Committee.

2.2. Study design

Sixty male apoE−/− mice (12–20 weeks old) were randomly divided into 3 groups, given rosuvastatin, atorvastatin (kindly provided by AstraZeneca and Pfizer Inc., respectively), or vehicle orally 7 days prior to initiation of AngII infusions and throughout the infusion period. Mice were visually inspected daily and weighed weekly. Rosuvastatin and atorvastatin were dissolved in drinking water at concentrations that gave approximate doses of 10 mg/kg/day and 20 mg/kg/day, respectively. Drug solutions were covered with foil to protect from light and freshly prepared every another day. Osmotic minipumps (Alzet Model 2004, Durect Corp) were implanted subcutaneously to deliver AngII (1000 ng/kg/min, Bachem) for 28 days. Suprarenal lumen dimensions were measured at selected intervals (Days 0 and 28) by a high frequency ultrasound imaging system (Visualsonics; Toronto, Canada) as described previously [16]. AAA was defined as an increase of 50% or greater in the maximal suprarenal diameter compared to the baseline.

2.3. Blood pressure measurement

Systolic blood pressure was measured in conscious mice using a computerized tail cuff (CODA 6+, Kent Scientific Corp, CT) [17]. All mice were acclimated to the system for 1 week prior to the start of the study.

2.4. AAA and atherosclerosis analyses

Mice were terminated after 28 days of AngII infusion, with blood harvested from left ventricles and aortic arches fixed with 10% neutral buffer formalin. Quantification of AAAs was based on luminal diameter and area as described above. Aneurysm severity was scored as described previously: Type I, dilated lumen without thrombus; Type II, remodeled tissue with little thrombus; Type III, a pronounced bulbous form of Type II with thrombus; Type IV, multiple, often overlapping aneurysms containing thrombus [17].

Aortic roots were embedded in OCT and frozen at −20 °C. Atherosclerosis was assessed on the intimas of both aortic arches by an en face technique and also using cross-sections (10 μm thick) of aortic roots as described previously [18]. Oil Red O staining was used to assist in visualization of lesions. Quantitative analysis of atherosclerosis was performed using Image-Pro software (Media Cybernetics) as described previously [1]. Cellular components of atherosclerotic plaques on aortic root were detected by immunostaining with rabbit antisera against mouse macrophage (Accurate Chemical Company), rabbit polyclonal to α-smooth muscle actin (α-SMA, Abcam), rat monoclonal to mouse CD19 for B lymphocyte (BD Pharmingen) and rat monoclonal to mouse CD90.2 for T lymphocyte (BD Pharmingen) [19]. Immunostaining was performed with a commercially available system (Fisher Microprobe). A peroxidase-based ABC system and the red chromogen, AEC, were used to visualize the antigen–antibody reaction. Cellular components consisting of atherosclerotic lesions were graded as follows: 0 = no staining, 1 = slight staining, 2 = mild staining, 3 = moderate staining and 4 = abundant staining (Supplemental data II Fig. 1). Six visual fields (magnification ×400) of every lesion section were randomly included to count the numbers of T lymphocytes and get the average of cell numbers in lesions.

Location of mRNA for nuclear factor κB (NF-κB p50, p65), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor (TNF-α), peroxisome proliferator-activated receptor (PPAR-α and -γ) in atherosclerotic lesions was determined with in situ hybridization kits and visualized using DAB as chromogen (Boster, Wuhan, China). Briefly, after fixed with 4% formaldehyde/PBS contained 0.1% DEPC, sections were quenched in freshly prepared 0.5% hydrogen peroxide in methanol, digested for 120 s at 37 °C in 10% (w/v) pepsin dissolved in 3% (w/v) citric acid, prehybridized in hybridization mix without probe for 2 h at 37 °C, and then hybridized overnight at 37 °C (probe sequence available on Supplemental data I). Probe bound to the section was immunologically detected using anti-digoxigenin Fab fragment covalently coupled to peroxidase and DAB as chromogenic substrate. All the data were quantified by two observers that were blinded to the study design.

Full details of the experimental protocols used are given in the Online supplemental data.

2.5. Serum measurements

Serum total cholesterol concentrations were determined using enzymatic assay kits (Wako Chemical Co). Serum concentrations of monocyte chemoattractant protein-1 (MCP-1, Bender MedSystems) and macrophage migration inhibitory factor (MIF, R&D Systems) were measured with ELISA kits according to manufacturer's recommendation. Serum concentrations of rosuvastatin and atorvastatin were measured by liquid chromatography with electrospray ionization tandem mass spectrometry as described previously [20]. Blood was collected about 1 h after drug administration to measure serum atorvastatin concentrations.

2.6. Statistical analyses

Mean and standard error of mean (SEM) were calculated for each parameter. Data were tested for use of parametric or non-parametric post hoc analysis and then analyzed by One way ANOVA

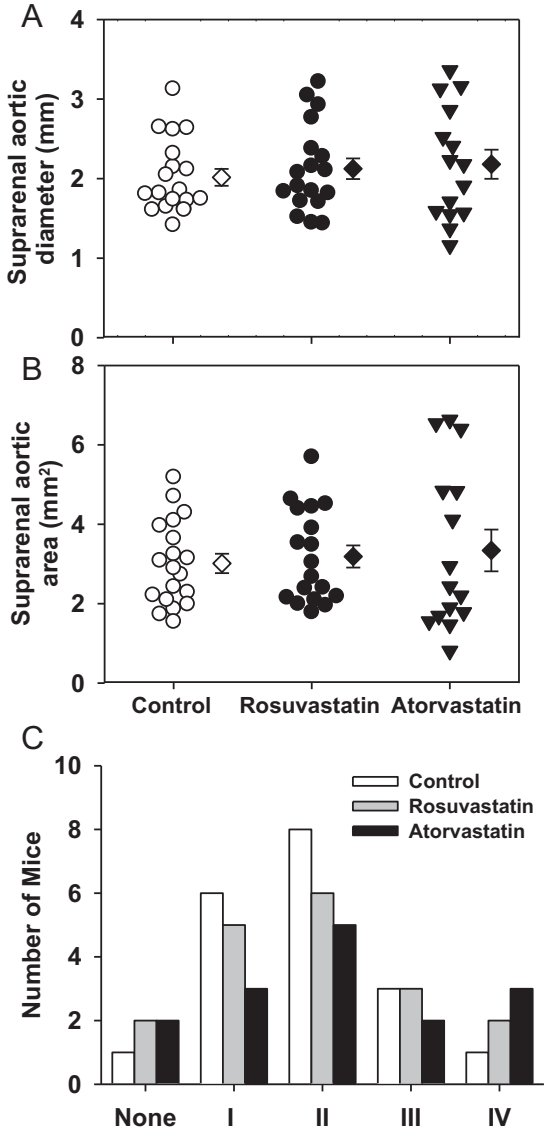

Fig. 1. Statins failed to suppress AngII-induced AAA. Maximal diameters (A) and areas (B) of suprarenal aortas were measured in apoE−/− mice infused with AngII for 28 days. Values of individual mice are represented as circles, diamonds are means, and bars are SEM. Classification of aneurysms (C) in mice infused with AngII alone or in combination with either rosuvastatin or atorvastatin.

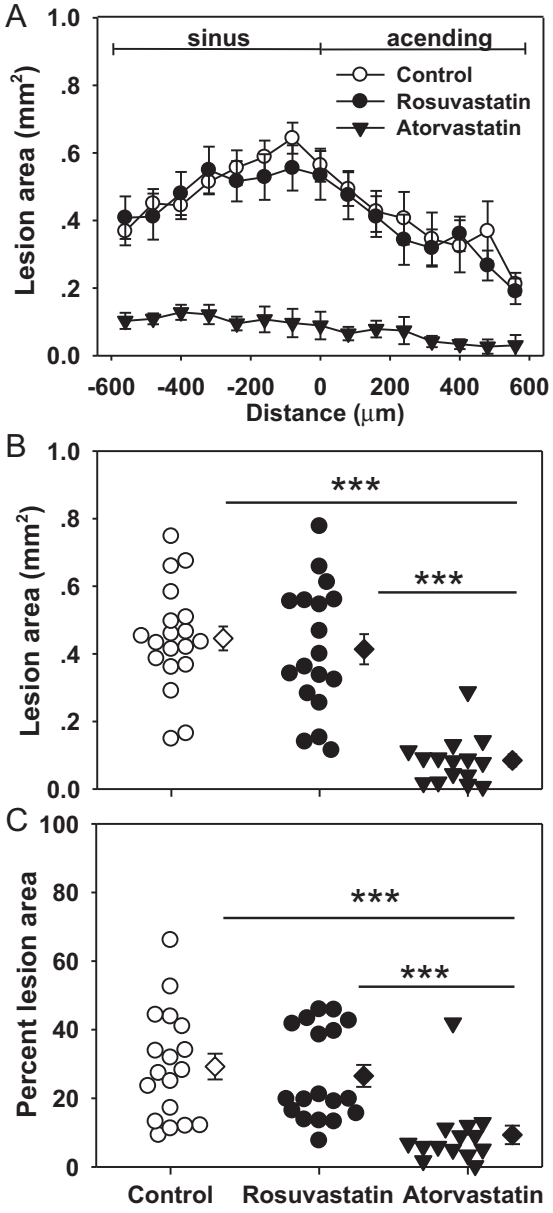

Fig. 2. Statins exerted differential effects on AngII-induced atherosclerosis. Measurement of atherosclerotic lesion size in apoE−/− mice infused with AngII for 28 days. (A) The distribution of lesion areas at 80 μm intervals in cross-sections of aortic roots from sinus to ascending aorta. (B) Mean atherosclerotic lesion areas in aortic root sections. (C) Percent of intimal area covered by atherosclerotic lesions in aortic arches. Values of individual mice are represented as circles, diamonds are means, and bars are SEM. ***$P < 0.001$, as determined by One way ANOVA.

or Dunn's test using SigmaStat v3.5 software. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Neither rosuvastatin nor atorvastatin altered development of AngII-induced AAAs

During the 28 days of AngII infusion, the aortic rupture incidence of AAA and thoracic aortic aneurysm (TAA) was 5–10%. These occurred between Day 7 and Day 10 in every group. There was no statistical significance of aneurysmal rupture among groups. After 28 days of AngII infusion, the AAAs incidence was 83% (17/20) in mice administered with rosuvastatin and 87% (13/15) in mice administered with atorvastatin, which was not significantly different from 95% (18/19) incidence of control mice. There was no statistical significance of suprarenal diameter or area among the different groups after 28 days of AngII infusion (Fig. 1). Based on the classification system of Daugherty [17], the majority of aneurysms were categorized in class II administered with rosuvastatin, atorvastatin or vehicle. Though more mice administered with atorvastatin developed Type IV aneurysms, there was no significant difference compared to mice administered with rosuvastatin or vehicle.

3.2. Rosuvastatin and atorvastatin had divergent effects on AngII-induced atherosclerosis

We determined the effects of rosuvastatin and atorvastatin in apoE−/− mice infused with AngII on atherosclerotic lesion areas in aortic roots (from aortic sinuses to ascending aorta) and percent of lesion areas on intimas of aortic arches. Administration of atorvastatin decreased lesion areas from aortic sinuses to ascending

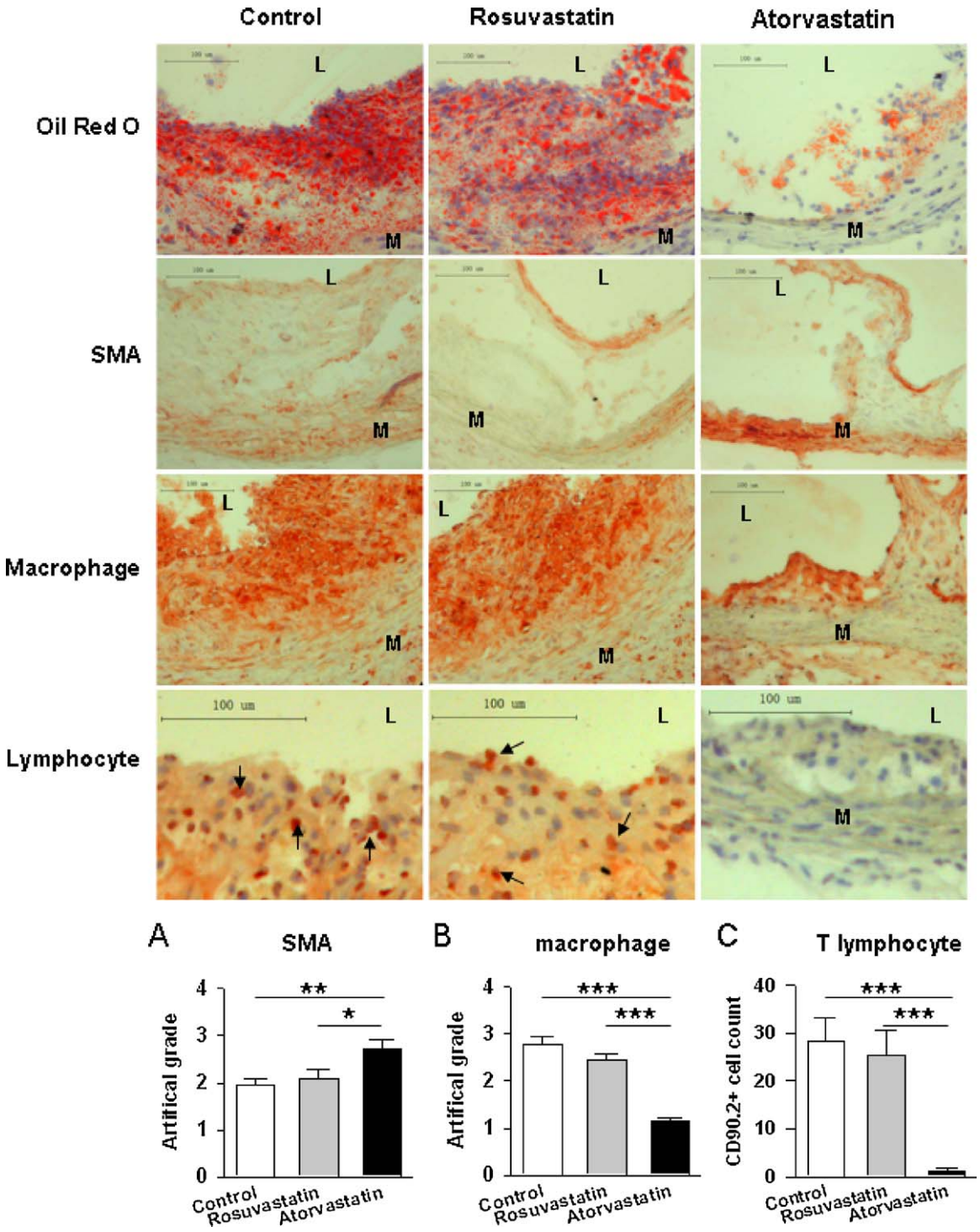

Fig. 3. Atorvastatin administration changed cellular compositions of lesions. Representative immunostaining for cellular composition of atherosclerotic lesions as shown in upper panel. Cellular components in lesions were analyzed by content-grading keys (4 grades) as shown in Supplemental data II Fig. 1, including (A) smooth muscle cells with rabbit polyclonal against α-smooth muscle actin and (B) macrophage with a rabbit anti-mouse serum against macrophages. The numbers of T lymphocytes (C) were counted randomly under microscope magnificated at X400. Representative T lymphocytes (CD90.2) were shown positive staining close to the nuclei below the endothelial surface of lesions (magnification shown in Supplemental data II Fig. 2) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as determined by One way ANOVA. Bar = 100 μm. L indicates lumen; M, media.

aortas compared to mice administered with rosuvastatin or vehicle (Fig. 2A and B, $P < 0.001$). Consistently, atorvastatin administration also profoundly decreased percent of lesion areas on intimas of aortic arches compared to mice administered with rosuvastatin or vehicle (Fig. 2C, $P < 0.001$).

To determine that therapeutic concentrations were achieved with the mode of delivery, rosuvastatin concentrations were detected in serum samples of experimental mice by liquid chromatography with electrospray ionization tandem mass spectrometry. Serum rosuvastatin concentrations were 41 ± 22 ng/ml in apoE−/− mice administered with rosuvastatin, while serum atorvastatin concentrations were 25 ± 9 ng/ml in apoE−/− mice administered with atorvastatin ($P < 0.001$). These are above the concentrations needed to inhibit mouse HMG-

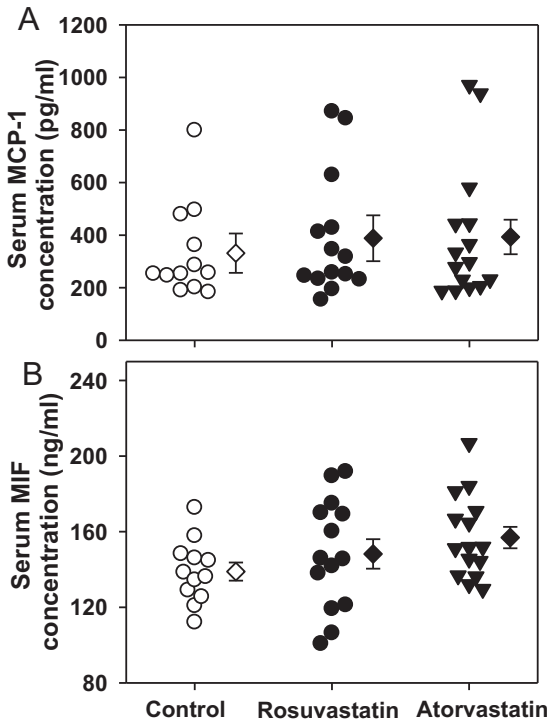

Fig. 4. Atorvastatin administration did not influence serum concentrations of inflammatory cytokines. Serum cytokine concentrations were measured by ELISA kits. (A) Serum MIF concentrations; (B) serum MCP-1 concentrations. Values of individual mice are represented as circles, diamonds are means, and bars are SEM.

CoA reductase [21,22]. No drug detected in serum of control mice.

3.3. Atorvastatin changed cellular composition of atherosclerotic lesions

Consistent to the previous reports [1], atherosclerotic lesions in apoE−/− mice infused with AngII have a characteristic of pronounced macrophages presented in the lesions and adventitia, a small number of T lymphocytes presented below the endothelial surface of lesions (magnification as shown in Supplemental data II Fig. 2), and weak expression of SMA in the media. B lymphocytes were not detected in the lesions of every group. Atorvastatin administration significantly increased the content of SMA in the lesions of mice, compared to vehicle or rosuvastatin administration (Fig. 3A, $P < 0.01$, $P < 0.05$, respectively). In contrast, administration of atorvastatin significantly decreased accumulation of macrophages and T lymphocytes in the lesions of mice compared to administered with vehicle or rosuvastatin (Fig. 3B and C, $P < 0.001$).

3.4. Atorvastatin altered inflammatory status in lesions, but not circulatory inflammatory cytokines

To investigate potential mechanisms of atorvastatin on attenuating atherosclerosis, serum MCP-1 and MIF concentrations were measured as an index of circulating inflammatory markers. AngII infusion did not influence serum concentrations of either MCP-1 or MIF ($P > 0.05$ by Student t test). Furthermore, serum concentrations of MCP-1 and MIF were not altered by either statin (Fig. 4). It is likely that circulating inflammatory markers were not reflective of local inflammatory status in atherosclerotic lesions. Therefore, we detected NF-κB, MCP-1, and TNF-α as major inflammatory factors in lesions using in situ hybridization. These were compared to PPAR α and γ as anti-inflammatory factor using this same technique. Although there were a low number of nuclei in lesion cores, NF-κB p50, p65, MCP-1 and TNF-α mRNA were diffusely presented in lesions, especially distributed in the luminal aspect and near the media. However, PPAR α and γ mRNA were localized at the boundary of the lesion intima and media in mice infused with AngII. Administration of atorvastatin dramatically attenuated NF-κB p50, p65, MCP-1 and TNF-α mRNA abundances in lesions of mice compared to administration of rosuvastatin or vehicle. In contrast, atorvastatin administration led to upregulation of anti-inflammatory PPAR-α and -γ mRNA abundances in lesions (Fig. 5). It is consistent with anti-atherosclerotic effect of atorvastatin being associated with alteration of inflammatory status within the lesions.

3.5. Atorvastatin exerted anti-atherosclerotic effect independent of lipid regulation

Consistent with previous reports [1], our studies demonstrated that AngII infusion did not change serum concentrations of total cholesterol (483 ± 42 mg/dl *vs* 453 ± 38 mg/dl). Moreover, administration of either rosuvastatin or atorvastatin did not alter serum total cholesterol concentrations (Supplemental data II Fig. 3). Neither statins altered body weight. AngII induced a significant increase of systolic blood pressure in apoE−/− mice that was not influenced by administration of either statin (Supplemental data II Fig. 4).

4. Discussion

In this study, we demonstrated that statin administration had no effect on AngII-induced AAAs, while having a differential role on AngII-augmented atherosclerosis. Atorvastatin administration not only reduced atherosclerotic lesion areas, but decreased macrophage and lymphocyte accumulation with an increase of SMA content in lesions. The anti-atherosclerotic effect of atorvastatin was associated with alterations of inflammatory status in lesions, independent of circulating concentrations of inflammatory cytokines and serum lipid regulations.

AngII induces AAA development that is associated with increasing vascular stiffness, promoting inflammation and proteolytic cascade. The rationale of the present study was based on reports from clinical trials that statin therapy reduced MMPs expression and activity in human aneurysmal walls to suppress AAA expansion [13]. Another study has demonstrated that atorvastatin administration exerted anti-inflammatory effects by inhibiting JNK signaling and dendritic cells recruitment [12]. However, the present study was not able to demonstrate beneficial effects of statins therapy on AngII-induced AAA, as reported for simvastatin [23]. Simvastatin administration was shown to inhibit angiogenesis and MMP secretion on AngII-induced AAA partly by inhibiting of ERK phosphorylation. This may indicate another therapeutic target beyond NF-κB signaling that mediates inflammatory genes expression and play an important role in formation of AngII-induced AAAs [24]. Though atorvastatin administration suppressed elastase-induced AAA by inhibiting macrophage recruitment, neither of statins in this study had any effect on AngII-induced AAA. It may implicate that various statins have divergent effects on AAA, and further studies are required to target pharmaceutical treatments.

Although statins therapy failed to suppress AngII-induced AAA, the study demonstrated differential effects against AngII-induced atherosclerosis of the two statins. AngII has been shown to induce macrophage HMG-CoA gene expression and increase cellular cholesterol biosynthesis, resulting in macrophage cholesterol accumulation and foam cell formation [25]. AngII infusion dramat-

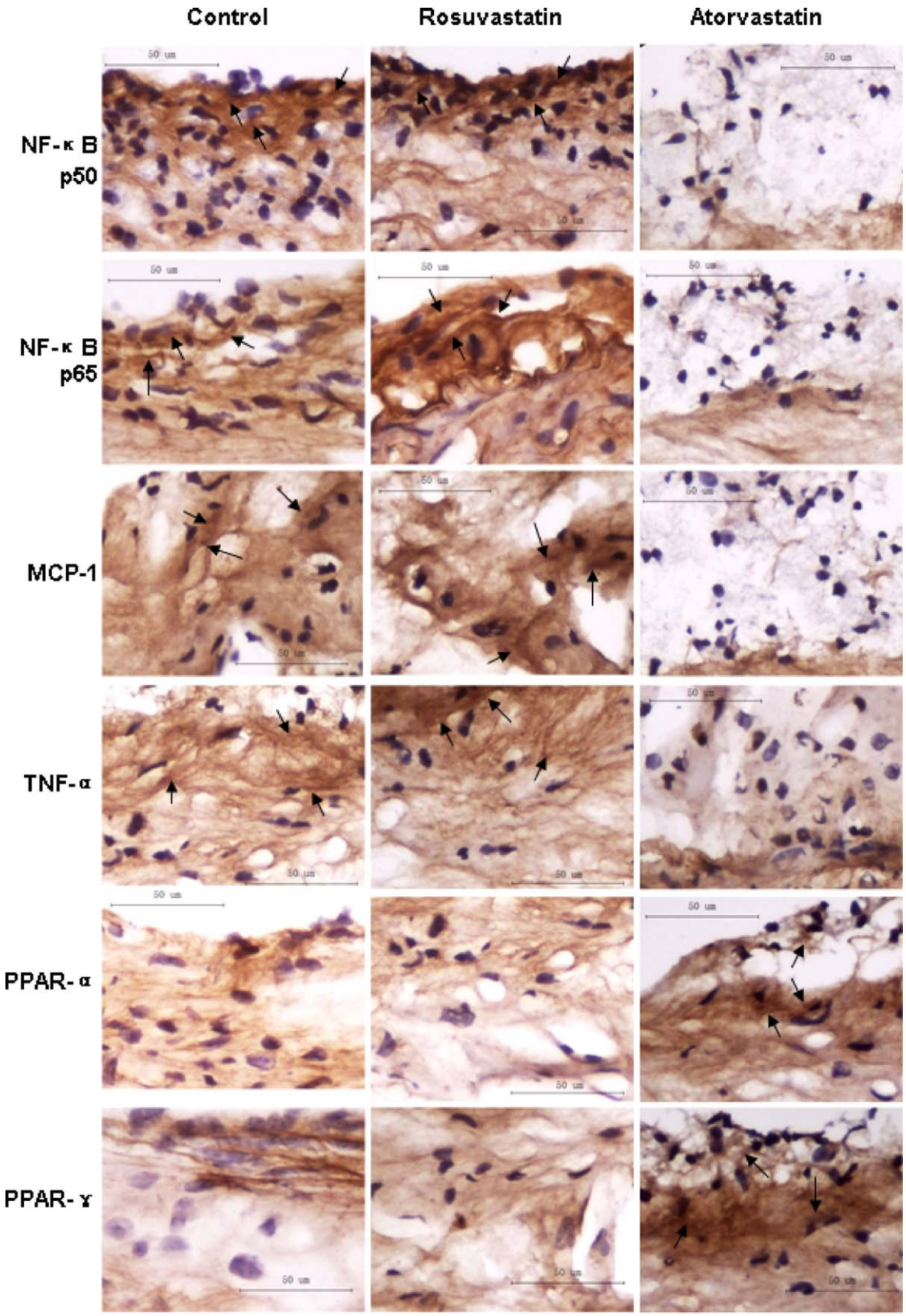

Fig. 5. Atorvastatin administration regulated inflammatory status in lesions. Representative mRNA abundance of NF-κB p50, NF-κB p65, MCP-1, TNF-α, PPAR-α and PPAR-γ in the cytoplasm (arrows) consisting of atherosclerotic lesions of apoE−/− mice administrated with vehicle, rosuvastatin or atorvastatin. Bar = 50 μm.

ically accelerates atherosclerosis in apoE−/− mice [1]. In contrast to previous report in apoE−/− mice [26], we also found that rosuvastatin administration had no effects on atherosclerosis during AngII infusion. However, administration of atorvastatin not only reduced lesion areas, but also altered cellular composition of atherosclerotic lesions. In addition, this study further supports the anti-atherosclerotic effects by statin therapy is independent of lipid regulations.

Leukocytic infiltration into the vasculature is crucial for AngII-induced AAA and atherosclerosis. It has suggested that MCP-1 functions as a central inflammatory mediator in the progression of AngII-induced atherosclerosis [27,28], and AngII stimulates MIF expression as well [29]. Although the differential effects of statins on atherosclerosis were found in this study, serum concentrations of MCP-1 and MIF were not affected by either AngII infusion or statin administration. It is consistent with circulating concentrations of inflammatory mediators not reflecting progression of AAA and atherosclerosis [30]. However, MCP-1 mRNA abundance was presented in atherosclerotic lesions of mice infused with AngII which was attenuated by administration of atorvastatin, suggesting that anti-atherosclerotic effect of atorvastatin was associated with inflammatory status in lesions. TNF is a potent chemoattractant for neutrophils that is produced by a broad variety of cells, mainly by macrophages. TNF-α can activate NF-κB by binding to TNF receptor and recruiting TRADD and other downstream adaptor proteins. We found that atorvastatin administration attenuated TNF-α mRNA abundance in lesions. Consistently, we showed that atorvastatin downregulated expression of NF-κB-mediated inflammatory genes, while upregulating expression of PPARs-mediated anti-inflammatory genes in lesions. The beneficial effects of atorvastastin may relate to being a lipophilic agent that freely penetrates cell membrane to regulate activity of inflammation-associated genes.

In summary, this study demonstrated that both rosuvastatin and atorvastatin administration failed to suppress development of AngII-induced AAAs. In contrast, atorvastatin reduced atherosclerosis associated with no change in circulating inflammatory markers but a shift to upregulation of anti-inflammatory genes and down-regulation of proinflammatory genes in the lesions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.03.005.

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