Atorvastatin promotes human monocyte differentiation toward alternative M2 macrophages through p38 mitogen-activated protein kinase-dependent peroxisome proliferator-activated receptor γ activation

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**A B S T R A C T**

M1 and M2 macrophages are detectable in human atherosclerotic lesions, and M2 macrophages are present at locations distant from the lipid core in more stable zones of the plaque and appear to exert anti-inflammatory properties on M1 macrophages. Peroxisome proliferator-activated receptor (PPAR) γ promotes the differentiation of monocytes into anti-inflammatory M2 macrophages. Although both statins and PPARγ ligands have been reported to protect against the progression of atherosclerosis, no data are currently available regarding the implication of statins in the alternative differentiation of human monocytes. In the present study, we hypothesized that atorvastatin may exert novel effects to prime human monocytes toward an anti-inflammatory alternative M2 phenotype. To this aim, we first found that abundant M2 markers were expressed in human circulating monocytes after atorvastatin treatment. Moreover, atorvastatin was able to induce PPARγ expression and activation in human monocytes in vivo and in vitro, resulting in priming primary human monocytes differentiation into M2 macrophages with a more pronounced paracrine anti-inflammatory activity in M1 macrophages. Additional data with molecular approaches revealed that p38 mitogen-activated protein kinase (MAPK) but not extracellular signal-regulated kinase (ERK) 1/2 activation was involved in atorvastatin-mediated PPARγ activation and enhanced alternative M2 macrophage phenotype. Collectively, our data demonstrated that atorvastatin promotes human monocyte differentiation toward alternative M2 macrophages via p38 MAPK-dependent PPARγ activation.

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1. Introduction

Atorvastatin, a widely used member of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have been shown to reduce the incidence of cardiovascular events and death in several mega trials [1–3]. The reasons why cardiovascular events were decreased with atorvastatin therapy are reported to be due to many pleiotropic effects, for instance, inhibition of the proliferation and migration of endothelial cells, smooth muscle cells, and macrophages [4].

Atherosclerosis is well established as a chronic inflammatory disorder involving many immune cells [5]. Among those cell types, macrophages were the first immune cells to be identified within atherosclerotic plaques [6], exerting an important impact on lesion progression in all stages of atherogenesis. A crucial step in this inflammatory process is the infiltration of monocytes into the subendothelial space of large arteries and their differentiation into tissue macrophages [7], which are a heterogeneous cell population adapting their activation state and functions to the microenvironment [8,9]. Therefore, macrophages exhibit a classical M1 activation profile in the presence of lipopolysaccharide (LPS) or interferon gamma (IFNγ). M1 macrophages are associated with inflammation and tissue destruction, produce proinflammatory cytokines such as TNF-α, interleukin (IL)-6, and monocyte chemotactic protein (MCP)-1 [10], and increase the production of reactive oxygen species sustaining the process of atherogenesis [11]. In contrast, IL-4 or IL-13 induced an alternative activation, known as M2. M2 macrophages dampen inflammatory process by producing anti-inflammatory mediators such as IL-10 and transforming growth factor-β, scavenging debris, and promoting angiogenesis, tissue remodeling and repair [10].

PPARγ (peroxisome proliferator-activated receptor) γ, a member of the nuclear hormone receptor family of ligand-dependent transcription factors, has been well characterized as potent anti-inflammatory properties that modulate the immune inflammatory response [12]. PPARγ is abundantly expressed in macrophages, where its expression is rapidly induced upon differentiation of monocytes into macrophages [13]. It is
demonstrated that M2 macrophages are present in human atherosclerotic lesions, where expression of M2 markers including CD206 and CCL-18 (chemokine (C–C motif) ligand 18) are positively correlated with the expression levels of the nuclear receptor PPARγ [14,15]. More importantly, monocytes can be primed by PPARγ activation to an enhanced anti-inflammatory M2 macrophages in atherosclerosis [14,16]. Interestingly, statins were reported to activate PPARγ in macrophages via extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (MAPK) activation and increase the DNA-binding activity of PPARγ to PPAR-response elements in monocytes [17]. However, it is not clear whether statins-induced PPARγ activation exerts novel effects to prime human monocytes toward an anti-inflammatory alternative M2 phenotype. If the hypothesis is true, the regulatory mechanisms of statin-induced PPARγ activation in monocytes are required to be further elucidated.

Therefore, in the present study, we first evaluated M2 markers expression in human circulating monocytes before and after atorvastatin treatment, and then investigated whether atorvastatin has the capacity to activate PPARγ to promote human monocyte differentiation toward alternative M2 macrophages and also to examine their underlying mechanisms. The data presented here demonstrated that atorvastatin was able to induce PPARγ expression and activation in human monocytes in vivo and in vitro, resulting in priming primary human monocytes differentiation into M2 macrophages with a more pronounced paracrine anti-inflammatory activity in M1 macrophages. Additional data with molecular approaches revealed that p38 MAPK but not ERK1/2 activation was involved in atorvastatin-mediated PPARγ activation and enhanced alternative M2 macrophage phenotype.

2. Materials and methods

2.1. Patients

Twenty non-diabetic patients with newly diagnosed coronary artery disease were enrolled from a single center and were treated with 20 to 40 mg atorvastatin p.o. daily. During the time they were receiving the atorvastatin, no other lipid lowering drugs were used. Peripheral venous blood (10 ml) was collected from each patient one day before and two months after atorvastatin treatment. This study was approved by the institutional ethics committee of First Hospital affiliated to Zhengzhou University (Henan, China), and written informed consent was obtained from every patient. The study was undertaken in full accordance with the Declaration of Helsinki, and other bioethical principles.

2.2. Cell preparation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from patients with newly diagnosed Coronary artery disease and from healthy donors by density gradient centrifugation using Ficoll–Hypaque as an established protocol [18]. Isolated PBMCs were washed twice and suspended in RPMI 1640 culture medium (GIBCO) supplemented with 10% (v/v) human serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were seeded at a density of 5 × 10^6 cells/well in six-well plates and incubated for 3 h in an atmosphere of 5% CO2 and 95% air. Nonadherent cells were discarded, and adherent monocytes were used for indicated tests or maintained in culture medium for 7 days to differentiate into resting macrophages (RM). RM were activated in M1 macrophages by the addition of LPS (100 ng/mL). Alternatively, differentiated macrophages (M2) were obtained by incubating freshly isolated monocytes with IL-4 (15 ng/ml) for 7 days. In some experiments, monocytes were coincubated for 7 days with IL-4 (15 ng/ml) and indicated concentrations of atorvastatin or its vehicle.

For the stimulation or inhibition assay, rosiglitazone (Sigma), T0070907 (Selleck Chemicals), PD98059 (Cell Signaling), and SB203580 (Sigma) were used.

2.3. Real-time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer’s instructions, and 1 μg of total RNA was converted to cDNA by SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies). PCR was performed on ABI Prism 7000 using corresponding primers and SYBR Green PCR Master Mix (Invitrogen). The primer sequences were provided in Supplementary Table 1. Template cDNA was denatured at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The quantification data were analyzed with ABI Prism 7000 SDS software. The cycle time values were normalized to GAPDH of the same sample. The expression levels of the mRNAs were then reported as fold changes versus the indicated control.

2.4. Flow cytometry

Monocytes were collected by gentle scraping and washed with ice-cold phosphate-buffered saline containing 1% BSA. The cells were then Fc-blocked by treatment with 1 μg of human IgG/10^5 cells for 30 min at 4 °C. Next, the cells were incubated at 4 °C for 30 min with monoclonal antibodies against CD206 (Allophycocyanin (APC), clone 685641, R&D systems) and CD163 (Phycoerythrin (PE), clone 215927, R&D systems) following the manufacturers’ instructions. Finally, the cells were washed and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). CD206 and CD163 expression levels are referred to as mean fluorescence intensity.

2.5. Enzyme-linked immunosorbent assay (ELISA)

2.5.1. ELISA for TNF-α and MCP-1

TNF-α and MCP-1 were measured in the supernatants using ELISA according to the manufacturer’s instruction (eBioscience).

2.5.2. ELISA for active PPARγ

The amount of activated PPARγ in nuclear extract (5 μg) was assessed by a sensitive ELISA assay for active PPARγ (TRANs-AM, Active Motif). The PPARγ ELISA kits contain a 96-well plate, which has been immobilized oligonucleotide containing the PPARγ consensus site (5′-AAGGTCAAAAGGTCA-3′). The active form of PPARγ contained in nuclear-cell extract specifically binds to this oligonucleotide. The primary antibodies used to detect PPARγ recognize an epitope on PPARγ that is accessible only when PPARγ is activated and bound to its target DNA. A horseradish peroxidase (HRP)-conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by spectrophotometry by reading within 5 min the absorbance at 450 nm.

2.5.3. ELISA for total and phosphorylated ERK and p38 MAPK

Total and phosphorylated ERK and p38 MAPK were measured using ERK1/2 (Total/Phospho (Thr202/Tyr204, Thr185/Tyr187)) InstantOne™ ELISA and p38 MAPK (Total/Phospho (Thr180/Tyr182)) InstantOne™ ELISA (eBioscience) separately following the manufacturers’ instructions. Cells were lysed in 100 μl per well of 1× Cell Lysis Mix (ELISA kit component) by incubation for 10 min at room temperature with shaking (300 rpm). Total soluble protein was quantified by BCA Protein Assay Kit and the same amount of protein was loaded per well (about 1 μg/well). Fifty microliters of antibody cocktail (capture antibody + detection antibody reagents from the ELISA kit) was added per well and incubated for 1 h at room temperature. After three washes with 200 μl 1 × wash buffer per well, 100 μL of Detection Reagent (kit component) was added per well and incubated for 30 min at room temperature. Finally, the reaction was terminated by adding 100 μl of Stop Solution (kit component) and the plate was read by measuring absorbance at 450 nm.
Whole-cell lysates were prepared and subjected to western blot analysis. Equal amounts of the cell lysates were resuspended in 5× Tris-glycine SDS sample buffer, electrophoresed on 12% SDS–PAGE, and transferred to nitrocellulose membranes (Amersham Pharmacia). The detection of proteins was performed with anti-phospho-p38 MAPK antibody, anti-Phospho-p38 MAPK (Thr180/Tyr182) antibody, anti-Phospho-Erk1/2 (Thr202/Tyr204, Thr185/Tyr187) antibody, anti-Erk1/2 antibody, and anti-3-actin antibody, followed by corresponding IDry second antibody. All antibodies were from Cell Signaling Technology. The blots were scanned using an Odyssey Imaging System (LI-COR Bioscience, USA).

2.7. Statistical analysis
Data analyses were performed using GraphPad Prism 5.0 software. Results are presented as mean ± standard deviation (SD). Differences between two groups were compared with the Student’s t-test. Intergroup comparison of means was performed by ANOVA followed by the Student’s t-test. P values less than 0.05 were considered to be significant.

3. Results

3.1. Abundant M2 markers are expressed in human peripheral blood monocytes after atorvastatin treatment

Previous studies have demonstrated that monocytes can be primed by PPARγ activation to an enhanced anti-inflammatory M2 macrophages in atherosclerosis [14]. To clarify whether atorvastatin treatment affects M2 markers expression in human peripheral blood monocytes, we performed M2 marker RNA analysis of monocytes isolated from patients before and after atorvastatin administration. Interestingly, atorvastatin treatment significantly increased the expression of the M2 markers such as CD206, IL-10 and CCL18 (Fig. 1A, C and D). However, atorvastatin therapy only caused a slight but not significant increment in the expression of the M2 marker CD163 (Fig. 1B), which was in line with previous reports of PPARγ agonists treatment [14]. Furthermore, the expression of PPARγ in circulating monocytes was also increased by atorvastatin treatment (Fig. 1E). This observation was concordant with the strikingly high amount of activated PPARγ protein detected in monocytes after atorvastatin therapy for two months (Fig. 1F). Taken together, these results suggest that atorvastatin treatment might be capable of priming monocytes cells toward an anti-inflammatory M2 phenotype through PPARγ activation in vivo.

3.2. Atorvastatin induces PPARγ expression and activation in human monocytes in vitro

We next examined the effect of atorvastatin on the PPARγ mRNA expression in isolated monocytes. Time course experiments for the PPARγ mRNA expression in monocytes revealed that atorvastatin (10 μM) significantly increased PPARγ mRNA expression levels by approximately 3-fold (versus 0 hour) at 12 and 24 h (Fig. 2A). As shown in Fig. 2B, the PPARγ mRNA expression levels were remarkably induced following atorvastatin treatment in a dose-dependent way. On the other hand, the nuclear expression of the activated PPARγ of monocytes dose escalation with atorvastatin treatment was also examined and found to be correspondent to the PPARγ mRNA expression (Fig. 2C). To further explore atorvastatin-mediated PPARγ activation, the mRNA expression of adipocyte fatty acid binding protein (aP2), a known PPARγ target gene in monocytes [19], was investigated. As shown in Fig. 2D, aP2 mRNA expression was also markedly induced in a dose-dependent manner by atorvastatin treatment. Furthermore, atorvastatin-induced PPARγ expression and activation were augmented by PPARγ agonist rosiglitazone and were reversed by PPARγ antagonist T0070907 (Fig. 2E-G).

3.3. Atorvastatin promotes alternative differentiation of human monocytes into macrophages via PPARγ activation and enhances anti-inflammatory properties

To determine whether atorvastatin influence M2 differentiation of human monocytes into macrophages, primary human monocytes were differentiated in vitro into alternative macrophages with IL-4 in
the presence of different concentrations of atorvastatin added only at the beginning of the differentiation process. As shown in Fig. 3A, the M2 marker CD206 was strongly induced after IL-4 stimulation and this effect was amplified by atorvastatin in a dose-dependent manner. By contrast, the expression of CD163 was significantly reduced by IL-4, which was consistent with previous studies [14,20,21]. Atorvastatin treatment enhanced the IL-4–induced decrease of CD163 expression in M2 macrophages. In addition, flow cytometry analysis demonstrated that the amount of both CD206 and CD163 on the surface of M2 macrophages was corresponded to their mRNA expression (Fig. 3B and C). Finally, coinucation of monocytes undergoing M2 differentiation with the PPARγ antagonist T0070907 completely abolished the regulation of CD206 and CD163 expression by atorvastatin, and an additive effect was observed with PPARγ agonist rosiglitazone treatment (Fig. 3D).

To determine whether atorvastatin–primed M2 macrophages can influence the inflammatory M1 macrophages, indirect coculture experiments were performed, and proinflammatory cytokines such as TNF-α and MCP-1 release by M1 macrophages were subsequently quantified. Incubation of M1 macrophages with medium from M2 macrophages–derived culture supernatant resulted in a pronounced inhibition of TNF-α and MCP-1 (Fig. 3E). Furthermore, this inhibitory effect was strongly augmented when atorvastatin were added at the beginning of the alternative differentiation of human monocytes into macrophages. Moreover, this inhibitory effect by atorvastatin was augmented by the PPARγ agonist rosiglitazone and completely reversed by the PPARγ antagonist T0070907 (Fig. 3E).

Collectively, these results suggested that atorvastatin–mediated promotion of human monocytes differentiation into M2 macrophages effects is PPARγ dependent.

3.4. p38 MAPK but not ERK1/2 activation is involved in atorvastatin–mediated PPARγ activation and enhanced alternative M2 macrophage phenotype

To further decipher the mechanism involved in atorvastatin–mediated PPARγ activation and enhanced alternative M2 macrophage phenotype, p38 MAPK and ERK1/2 phosphorylations by atorvastatin treatment were assessed. As shown in Fig. 4A and B, atorvastatin could markedly induced phosphorylation of p38 MAPK, however, only caused a slight but not significant ERK1/2 activation. Western blot analysis also confirmed the results of ELISA tests (Fig. 4C and D). Furthermore, both atorvastatin–induced PPARγ expression and activation were abolished by p38 MAPK–specific inhibitor SB203580 in a dose–dependent way, whereas no obvious changes were found after MAPK/ERK kinase–specific inhibitor PD98059 treatment (Fig. 4E and F). Most importantly, SB203580 but not PD98059 treatment blocked PPARγ dependent M2 macrophage differentiation by altering the expressions of CD206 and CD163 in both mRNA and protein levels (Fig. 4G and H).

4. Discussion

Monocytes are precursors of macrophages, which are prominent cells in the response to lipid accumulation in large arteries that contribute to the development of atherosclerosis and its complications. It is characterized that monocytes preferentially migrate to lesions with high inflammatory activity and to give rise to the macrophages in atherosclerotic lesions in mice [22,23], indicating that circulating monocytes have the capacity to influence plaque formation. It has been also reported that the expression of PPARγ positively correlates with expression levels of M2 markers, such as CD206, CCL18, and IL-10 in human atherosclerotic plaques [14,16]. Besides, Pucci et al. demonstrated that PPARγ expression was higher in coronary plaques and peripheral blood monocytes of statin–treated patients [15]. In the current study, abundant M2 markers such as CD206, IL-10, and CCL18 were expressed in human circulating monocytes after atorvastatin treatment. In fact, the expression of PPARγ gene positively correlates with mRNA levels of M2 markers, such as CD206, CCL18, and IL-10 in peripheral blood monocytes of patients in the current study (data not shown). Notably, we also found that increasing expression of PPARγ in mRNA level and amount of activated PPARγ protein were detected in monocytes after atorvastatin administration for 2 months. Previous reports have shown that statins activate PPARγ in monocytes as well as macrophages [17,24]. Consistent with the previous results, our results confirmed that atorvastatin dose dependently induced PPARγ expression and activation in human monocytes in vitro. Taken together, these results demonstrated that atorvastatin–mediated PPARγ activation in vivo and in vitro, implicating that atorvastatin might be able to prime monocytes cells toward an anti–inflammatory M2 phenotype via PPARγ activation.
Fig. 3. Atorvastatin promotes alternative differentiation of human monocytes into macrophages via PPARγ activation and enhances anti-inflammatory properties. Primary human monocytes were differentiated to M2 in the presence or absence of 10μM atorvastatin added only at the beginning of the differentiation process. (A) Real-time PCR analysis of CD206 and CD163 mRNA in RM and M2 macrophages. (B) Flow cytometry analysis of CD206 and CD163 on the surface of RM and M2 cells. Data are referred to as mean fluorescence intensity $P < 0.05$ for M2 versus RM. $*P < 0.05$ for indicated concentrations of atorvastatin versus vehicle in M2. (C) Representative figures of flow cytometry. (D) Primary human monocytes were differentiated to M2 in the presence or absence of T0070907 (10 nM) or rosiglitazone (100 nM) added only at the beginning of the differentiation process. Real-time PCR analysis of CD206 and CD163 mRNA in RM and M2 macrophages treated with 10μM atorvastatin or vehicle. $P < 0.05$ for atorvastatin versus vehicle. $*P < 0.05$ for atorvastatin plus the indicated inhibitor versus atorvastatin. (E) TNF-α and MCP-1 were quantified in macrophage supernatants from M1 macrophages, which had been previously exposed to medium from atorvastatin primed-M2 macrophage cultures with or without T0070907 (10 nM) or rosiglitazone (100 nM) added only at the beginning of the differentiation process. $P < 0.05$ for atorvastatin versus vehicle. $*P < 0.05$ for atorvastatin plus the indicated chemicals versus atorvastatin. Columns, mean $(n = 6)$; bars, SD.
Since the presence of both M1 and M2 macrophage markers has been demonstrated in human atherosclerotic lesions, the concept of macrophage heterogeneity has entered the field of atherosclerosis research [25,26]. M2 macrophages are present at locations distant from the lipid core in more stable zones of the plaque and appear to exert anti-inflammatory properties on M1 macrophages. Intriguingly, PPARγ expression in anti-inflammatory macrophages positively correlates with the expression of M2 markers [14]. In our hands, atorvastatin was also shown to induce PPARγ expression and activation in human monocytes in vivo and in vitro, thus prompted us to examine whether atorvastatin plays a role in the differentiation program of monocytes into the M2 phenotype. As hypothesized, monocytes could be primed by atorvastatin toward an enhanced M2 phenotype in the presence of IL-4 stimuli. Furthermore, atorvastatin-activated M2 macrophages dampened the inflammatory status of surrounding M1 macrophages and thus exerted a paracrine anti-inflammatory effect on M1 macrophages. In addition, these phenomena were augmented by PPARγ agonist rosiglitazone and completely reversed by PPARγ antagonist T0070907, supporting the notion that an additional mechanism by which atorvastatin can exert anti-inflammatory activities in macrophages mainly via PPARγ activation. Most importantly, this presents yet another molecular pathway through which statins exhibit its anti-inflammatory effects upon the vasculature, inhibiting plaque formation and stabilizing established atherosclerotic plaques [27,28].

As discussed above, the mechanism via which atorvastatin promotes monocytes toward the M2 phenotype involves PPARγ activation. In fact, PPARγ is negatively regulated by mitogen-activated protein kinase (MAPK) via its phosphorylation [29]. However, atorvastatin does not induce serine phosphorylation of PPARγ in human monocyte/macrophage (THP-1) cells [30]. Thus, atorvastatin-induced PPARγ activation cannot be mediated by inhibition of MAPK-dependent serine phosphorylation.
Previous results also note that statins activate ERK1/2 and p38 MAPK to induce PPARγ activation in murine macrophages [17]. Nevertheless, our results proved that atorvastatin significantly induced phosphorylation of p38 MAPK while sparing ERK1/2 phosphorylation in human monocytes. Moreover, both atorvastatin-induced PPARγ expression and activation were abolished by p38 MAPK-specific inhibitor SB203580, whereas no significant changes were found in MAPK/ERK kinase-specific inhibitor PD98059 treatment. Most importantly, SB203580 but not PD98059 treatment blocked atorvastatin priming monocytes cells toward an anti-inflammatory M2 phenotype. The underlying mechanisms behind why atorvastatin only activated p38 MAPK but not propelled ERK1/2 phosphorylation to cause PPARγ activation and thus enhance alternative M2 macrophage phenotype remains unknown.

In conclusion, we first found that abundant M2 markers were expressed in human circulating monocytes after atorvastatin treatment. Moreover, atorvastatin was able to induce PPARγ expression and activation in human monocytes in vivo and in vitro, resulting in priming primary human monocytes differentiation into M2 macrophages with a more pronounced paracrine anti-inflammatory activity in M1 macrophages. Additional molecular approaches revealed that p38 MAPK but not ERK1/2 activation was involved in atorvastatin-mediated PPARγ activation and enhanced alternative M2 macrophage phenotype. Our data provide additional insight into how atorvastatin might be involved in attenuating vascular inflammation and atherosclerosis, at least in part, lead to generation of a macrophage population with enhanced anti-inflammatory properties and highlight an entirely novel biological basis for the atheroprotective effects in atorvastatin treatment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2015.03.005.

Conflict of interest

The authors have no conflict of interest.

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