

Rosuvastatin promotes the differentiation of peripheral blood monocytes into M2 macrophages in patients with atherosclerosis by activating PPAR- γ

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Abstract. – **OBJECTIVE:** To evaluate M2 marker changes in human circulating monocytes before and after rosuvastatin treatment, and to investigate the effects of rosuvastatin on the differentiation of monocytes into M2 macrophages by activating peroxisome proliferator-activated receptor- γ (PPAR- γ).

PATIENTS AND METHODS: A total of 20 patients was administrated with rosuvastatin. The human peripheral blood mononuclear cells (PBMCs) were extracted by Ficoll-Hypaque density gradient centrifugation method. PPAR- γ , CD206 and CD163 mRNA levels were detected by Real-time polymerase chain reaction (RT-PCR). The total content of tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), PPAR- γ , extracellular signal-regulated kinase (ERK) and p38 Mitogen-activated protein kinase (MAPK) and the contents of phosphorylated ERK and p38 MAPK were determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS: The expression levels of CD206, Interleukin 10 (IL-10), and chemokine (C-C motif) ligand 18 (CCL18) were significantly improved by rosuvastatin. The expression level of PPAR- γ in circulating monocytes was also distinctly up-regulated through the treatment with rosuvastatin. After rosuvastatin therapy, PPAR- γ mRNA expression was unceasingly increased with time prolonging. The tendency of mRNA level of p38 was the same as that of PPAR- γ . In vitro experiments indicated that in M2 macrophages, rosuvastatin could enhance the decrease of CD163 expression level induced by interleukin 4 (IL-4). M1 macrophages cultured by supernatant that was used to culture M2 macrophages could significantly inhibit TNF- α and MCP-1 expressions. Rosuvastatin could remarkably induce the phosphorylation of p38 MAPK, but the effect on ERK1/2 was not obvious.

CONCLUSIONS: Our results confirmed expressions of M2 markers in human circulating peripheral blood monocytes after rosuvastatin therapy. Both in vivo and in vitro experiments proved that rosuvastatin can induce the expres-

sion and activation of PPAR- γ in human monocytes, resulting in the differentiation of monocytes into M2 macrophages.

Key Words:

Rosuvastatin, Atherosclerosis, PPAR- γ , Monocytes, M2 macrophages.

Introduction

Atherosclerosis is a kind of arteriosclerosis and the most important form of vascular diseases. The present work reveals that atherosclerosis is a chronic inflammatory disease involving a variety of immune cells^{1,2}. Macrophages are the first identified inflammatory cells associated with atherosclerotic plaques and have an important influence on the development of lesions in the whole process of atherosclerosis^{3,4}. An important step in the development of inflammation is the infiltration of monocytes into the subcutaneous space of large arteries and the differentiation into different macrophages. The direction of cell differentiation mainly depends on the state of cell activation and surrounding microenvironment. When lipopolysaccharide (LPS) or interferon- γ (IFN- γ) exists, monocytes tend to differentiate into M1 macrophages. M1 macrophages are related to inflammation and tissue damage, which can secrete proinflammatory cytokines such as tumor necrosis factor (TNF- γ), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), and can increase the generation of active oxygen to maintain the formation of atherosclerosis⁵⁻⁷. On the contrary, when there is interleukin-4 (IL-4) or interleukin-3 (IL-13), monocytes tend to differentiate into M2 macrophages. M2 macrophages can inhibit inflammatory process, remove debris

and promote angiogenesis and tissue repair and reconstruction via producing IL-10 and transforming growth factor- β ^{8,9}. M2 macrophages also exist in the human atherosclerotic plaques¹⁰.

Peroxisome proliferator-activated receptor- γ (PPAR- γ) is a ligand-activated receptor in the nuclear hormone receptor family. The study indicates that PPAR- γ has anti-inflammatory activity that can regulate immune inflammatory reaction^{11,12}. When monocytes are differentiated into macrophages, PPAR- γ can be largely expressed in macrophages, and its expression level is closely correlated with the contents of M2 macrophage markers, CD206 and chemokine (C-C motif) ligand 18 (CCL18). More importantly, in atherosclerotic lesions, monocytes can be activated by PPAR- γ to differentiate into enhanced anti-inflammatory M2 macrophages^{13,14}.

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, statins, is a kind of prevention and treatment drug for coronary heart disease, hypertension and cerebrovascular disease. As a new type of lipid-lowering drugs, rosuvastatin is widely applied in clinical practice. Apart from the strong lipid-lowering effect, it is of great importance in inhibiting inflammatory reaction, ameliorating vascular endothelial function and stabilizing plaque. The study shows that statins can activate PPAR- γ in macrophages¹⁵, which is achieved mainly via activating extracellular signal-regulated protein kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (MAPK) and enhancing DNA binding activity of PPAR- γ on PPAR response element¹⁶. However, it is not clear whether PPAR- γ activation induced by statins affects the differentiation of human monocytes into anti-inflammatory M2 macrophages; if the effect exists, its mechanism remains to be intensively investigated.

The primary purpose of this investigation was to evaluate M2 marker changes in human circulating monocytes before and after rosuvastatin treatment, and to investigate the effects of rosuvastatin on the differentiation of monocytes into M2 macrophages by activating peroxisome proliferator-activated receptor- γ (PPAR- γ).

Patients and Methods

Data of Patients

The enrolled 20 patients admitted to Suzhou Kowloon hospital without diabetes mellitus and diagnosed as coronary artery disease were ad-

ministrated with rosuvastatin (dose within 10-20 mg). This study was approved by the Ethics Committee of Suzhou Kowloon hospital. Signed written informed consents were obtained from all participants before the study. During this period, patients were treated with rosuvastatin only, without taking other lipid-lowering drugs. 10 mL peripheral venous blood was collected from patients on the day before treatment and at two months after treatment, respectively.

Cell Preparation and Culture

Human peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation method. The specific steps are shown as follows: 10 mL venous blood was collected and added with the equal volume of phosphate buffered saline (PBS) at room temperature, so as to dilute the blood for equal times. The layered liquid of Ficoll-Hypaque (5 mL layered liquid for each 10 mL diluted blood) was added, followed by being placed into a 50 mL centrifuge tube. After centrifugation, the solution in the tube could be divided into four layers. The turbid or white layer at the junction of layered liquid and plasma was mononuclear cell layer. The white blood mononuclear cells were slightly absorbed by capillary pipette and placed into another centrifuge tube for reservation. Then, it was washed by Roswell Park Memorial Institute-1640 (RPMI-1640) for two times and suspended in RPMI-(1640) nutrient solution containing 10% human serum, penicillin (100 U/mL) and streptomycin (100 μ g/mL). The cells were placed into a 6-well plate and cultured in an incubator containing 5% CO₂ and 95% air for 3 h. The non-adherent cells were discarded, and the remaining cells were selected as control or cultured in the cell nutrient solution for 7 days to differentiate monocytes. Lipopolysaccharide (LPS) (100 ng/mL) was added to a portion of cells to promote the differentiation of monocytes into M1 macrophages, while IL-4 (15 ng/mL) was added to another cells to induce the differentiation of cells into M2 macrophages.

Real-time Fluorescence Quantitative Polymerase Chain Reaction

RNA was extracted by TRIzol method, and cDNA was synthesized via reverse transcription by RT-PCR. First, 40 cycles were conducted for template cDNA, and the results were analyzed by a fluorescent quantitative PCR instrument. In order to further verify PPAR- γ expression and

activation induced by PPAR- γ , PPAR- γ agonist (100 nM) and PPAR- γ antagonist T0070907 (10 nM) were joined (or not) in this study. Meanwhile, in order to investigate PPAR- γ activation induced by rosuvastatin and differentiation mechanism of monocytes into M2 macrophages, p38 MAPK specific inhibitor SB203580 and MAPK/ERK specific inhibitor PD98059 were respectively added in this study.

Flow Cytometry

Monocytes were rinsed by precooling phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) for two times. 1 μ g IgG was added in each 10⁵ cells, followed by placing at 4°C for 30 min, for sealing the possible Fc receptor. Anti-CD206 monoclonal antibody and anti-CD163 monoclonal antibody were added to cells, and the expression levels of CD206 and CD163 were detected by flow cytometer (Partec AG, Arlesheim, Switzerland).

ELISA

The supernatant used to culture M2 macrophages was utilized to culture M1 macrophages, followed by detecting TNF- α and MCP-1 using ELISA. M2 macrophages were treated with PPAR- γ agonist (100 nM) or antagonist (10 nM). Subsequently, the cultured supernatant of M1 macrophages was taken for detecting TNF- α and MCP-1 contents. The specific process was carried out in accordance with the ELISA test kit (eBioscience Inc., San Diego, CA, USA).

Western Blot

Cell lysates were analyzed using Western blot. The lysate was suspended in 5 \times Tris-glycine sodium dodecylsulfate (SDS) buffer, followed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, protein was transferred onto the nitrocellulose membrane. Then, the used primary antibodies, anti-p38 MAPK antibody, anti-phosphorylated p38 MAPK (Thr180/Tyr182) antibody, anti-phosphorylated Erk1/2 (Thr202/Tyr204, Thr185/187) antibody, anti-Erk1/2 antibody and anti- β -actin antibody were respectively utilized to detect the corresponding proteins.

Statistical Analysis

The data were analyzed by GraphPad Prism 6.0 software (La Jolla, CA, USA) and the results were represented by mean \pm standard deviation. The *t*-test was used for intergroup comparison,

and the analysis of variance (ANOVA) and *t*-test were adopted for comparison in each group. *p* < 0.05 suggested that the difference was statistically significant.

Results

Rosuvastatin Significantly Increases the Expression Levels of PPAR- γ and M2 Markers

To understand whether rosuvastatin affects the expressions of M2 markers in human peripheral blood monocytes, the peripheral blood was respectively extracted from patients before and after receiving rosuvastatin. The contents of M2 marker RNA in monocytes were detected by Real-time polymerase chain reaction (RT-PCR). The results displayed that rosuvastatin could significantly increase the expression levels of M2 markers including CD206, IL-10 and CCL18 (Figure 1A-C). Additionally, the expression level of PPAR- γ mRNA in circulating monocytes was also significantly up-regulated after rosuvastatin treatment, which was further confirmed by ELISA results (Figure 1D-E).

In Vitro Experimental Results of PPAR- γ Expression and Activation Induced by Rosuvastatin

Through the treatment with 10 μ M rosuvastatin, the expression of PPAR- γ mRNA was unceasingly increased with time prolonging, which was basically stable at 12 h and 24 h (Figure 2A). PPAR- γ mRNA expression level was in a dose-dependent manner, and the larger the dose of rosuvastatin was, the more the PPAR- γ mRNA expression level would be, which had significant differences among different doses (Figure 1B). The tendency of activated PPAR- γ expression level was the same as that of PPAR- γ mRNA (Figure 1C). As a kind of PPAR- γ target gene in monocytes, the content of adipocyte fatty acid-binding protein was determined. As shown in the Figure 2D, aP2 mRNA levels were also dose-dependent with rosuvastatin, which was the same as the tendency of PPAR- γ mRNA. To further verify PPAR- γ expression and activation induced by PPAR- γ , PPAR- γ agonist (100 nM) and PPAR- γ antagonist T0070907 (10 nM) were further joined in this study. Through the treatment with 10 μ M rosuvastatin for 24 h, the expression levels of PPAR- γ mRNA, activated PPAR- γ , and aP2 mRNA, were affected by PPAR- γ agonist and antagonist (Figure 2EFG).

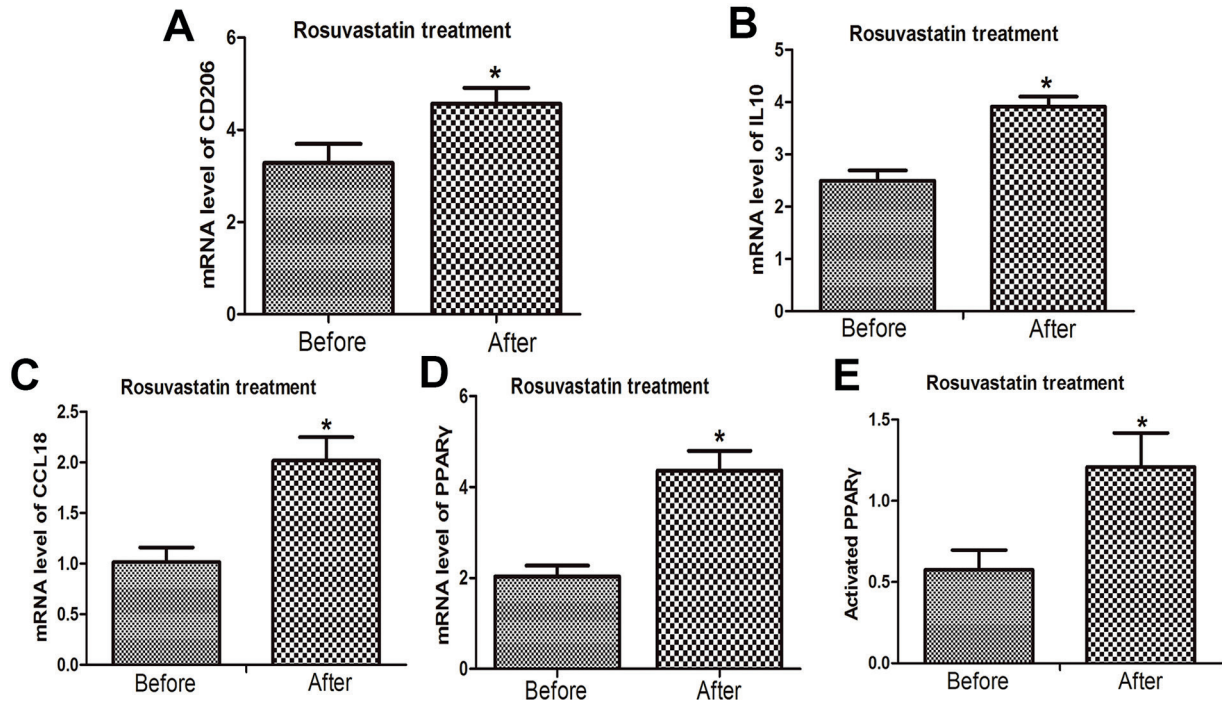


Figure 1. Rosuvastatin significantly increases the expression levels of M2 markers and PPAR- γ . **(A)** Relative mRNA level of CD206 before and after rosuvastatin treatment. **(B)** Relative mRNA level of IL-10 before and after rosuvastatin treatment. **(C)** Relative mRNA level of CCL18 before and after rosuvastatin treatment. **(D)** Relative mRNA level of PPAR γ before and after rosuvastatin treatment. **(E)** Activated PPAR γ detected by ELISA before and after rosuvastatin treatment; * $p < 0.05$.

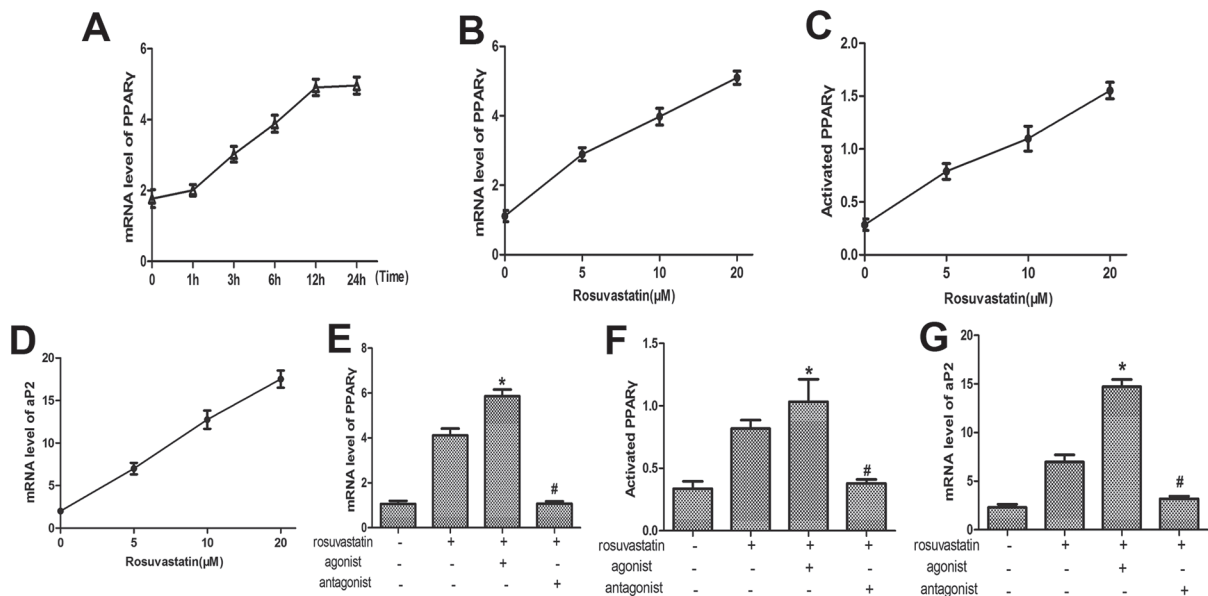


Figure 2. *In vitro* experimental results of PPAR- γ expression and activation induced by rosuvastatin. **(A)** The mRNA level of PPAR- γ at different time after treatment of 10 μ M rosuvastatin. **(B)** The mRNA level of PPAR- γ after treatment of different concentrations of rosuvastatin. **(C)** Activated PPAR- γ detected by ELISA after treatment of different concentrations of rosuvastatin. **(D)** The mRNA level of aP2 after treatment of different concentrations of rosuvastatin. **(E, F, G)** The expression levels of PPAR- γ mRNA, activated PPAR- γ and aP2 mRNA were affected by PPAR- γ agonist and antagonist. * $p < 0.05$ vs. Control group, # $p < 0.05$ vs. Control group.

Detection of CD206 and CD163 Expression Levels

M2 marker, CD206, was significantly increased with the stimulation by IL-4, which was amplified with the increased dose of rosuvastatin, indicating that the expression level of CD206 mRNA was dose-dependent with rosuvastatin (Figure 3A). On the contrary, CD163 content was inhibited by IL-4, which was enhanced after adding rosuvastatin (Figure 3B). The conclusion was also confirmed by the results of flow cytometry (Figure 3C-D). After adding PPAR- γ antagonist T0070907, the regulation of rosuvastatin on the expressions of CD206 and CD163 was completely inhibited, which was enhanced by adding PPAR- γ agonist (Figure 2E-F). Meanwhile, in order to explore whether the differentiation of monocytes into M2 macrophages induced by rosuvastatin affects M1 macrophages, the contents of proinflammatory cytokines such as TNF- α and MCP-1 secreted by M1 macrophages were detected by the indirect co-culture test in this study. M1 macrophages cultured by supernatant that was used to culture M2 macrophages could significantly inhibit TNF- α and MCP-1 expressions. If rosuvastatin was added at the beginning of differentiation, the inhibitory effect would be increased significantly, but if PPAR- γ agonist and antagonist were added, it would be enhanced or decreased accordingly (Figure 2G-H).

Activation of PPAR- γ Induced By Rosuvastatin and Pathway of Monocytes Tending to Differentiate Into M2 Macrophages

Rosuvastatin could significantly induce p38 MAPK phosphorylation, but the effect on ERK1/2 was not obvious (Figure 4A), which was confirmed by Western blot results (Figure 4B). Additionally, PPAR- γ expression and activation induced by rosuvastatin could be significantly inhibited by p38 MAPK specific inhibitor SB203580, which was in a dose-dependent manner. The inhibitory effect was not obvious when MAPK/ERK specific inhibitor PD98059 was added (Figure 4C-D). Similarly, the detection results of CD206 and CD163 mRNA levels also revealed that the differentiation of M2 macrophages that depended on PPAR- γ was blocked by SB203580, not PD98056 (Figure 4E).

Discussion

Monocytes are precursors of macrophages and a kind of important responder cells of accumulation of fat in the large human arteries. The accumulation of fat in the large arteries may cause atherosclerosis and complications. The study shows that monocytes first migrate to the lesions with an inflammatory activity and

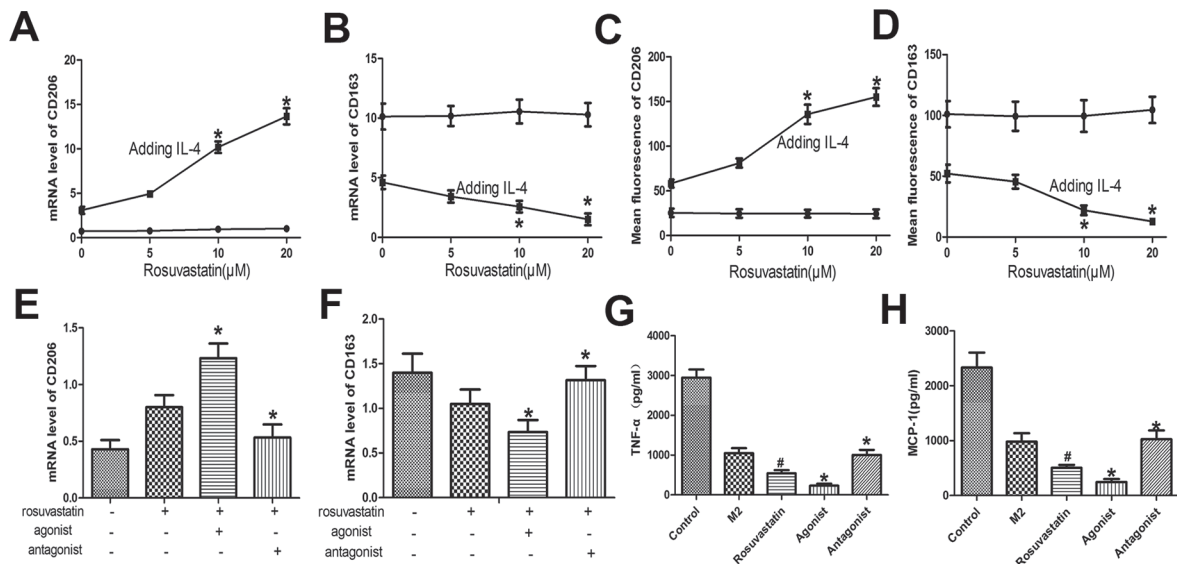


Figure 3. Detection of CD206 and CD163 expression levels. **(A, B)** The mRNA level of CD206 and CD163 after treatment of different concentrations of rosuvastatin. **(C, D)** Detection of CD206 and CD163 expression levels by flow cytometry. **(E) (F)** The mRNA level of CD206 and CD163 after treatment of PPAR- γ agonist and antagonist. **(G, H)** The level of TNF- α and MCP-1 secreted by M1 macrophages under different conditions. * $p < 0.05$.

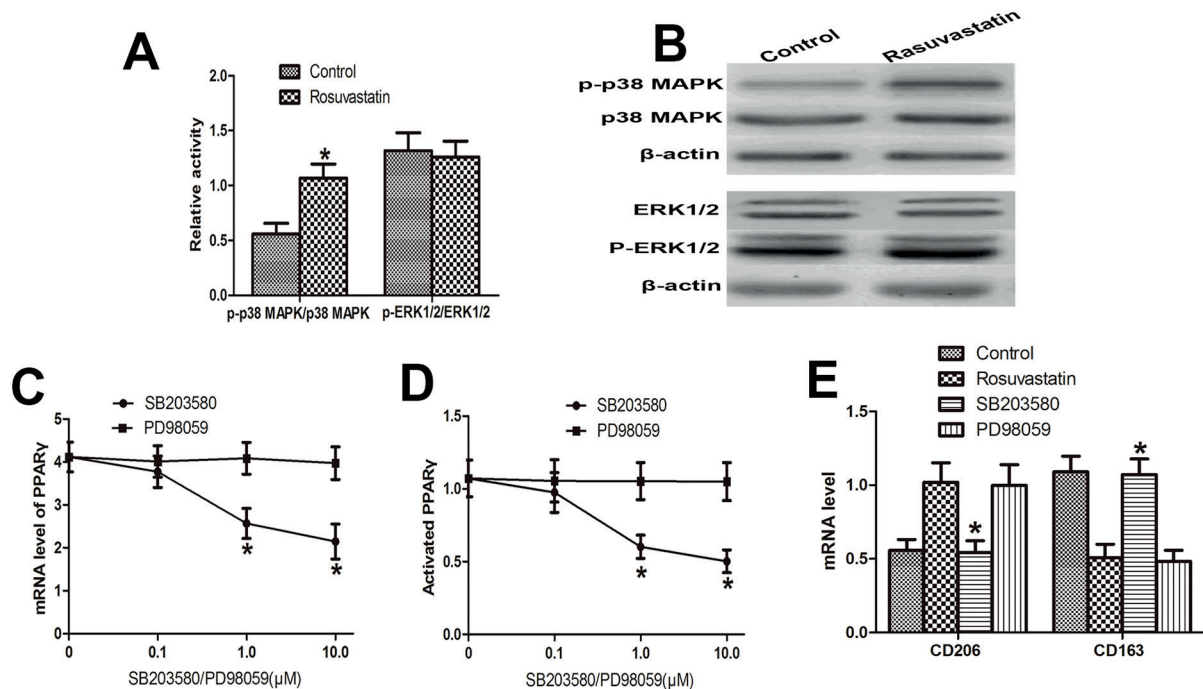


Figure 4. Activation of PPAR- γ induced by rosuvastatin and pathway of monocytes tending to differentiate into M2 macrophages. **(A)** The level of p-p38 MAPK and p-ERK1/2 detected by ELISA before and after rosuvastatin treatment. **(B)** Western blot analysis reveals the expression of p-p38 MAPK and p-ERK1/2. **(C, D)** The mRNA and activation of PPAR- γ affected by p38 MAPK specific inhibitor and MAPK/ERK specific inhibitor. **(E)** The mRNA level of CD206 and CD163 affected by p38 MAPK specific inhibitor and MAPK/ERK specific inhibitor. * $p < 0.05$.

differentiate into macrophages, indicating that circulating monocytes can affect the formation of atherosclerotic plaques³. Additionally, the study reveals that in human atherosclerotic plaques, PPAR- γ expression level is closely related to the expression levels of M2 macrophage markers such as CD206, CCL18 and IL-10¹⁷. PPAR γ is high expressed in coronary artery plaque and peripheral blood mononuclear cells of patients receiving statin therapy¹⁸. The recent study displays that the expression levels of M2 markers such as CD206, IL-10 and CCL18 are markedly increased in human circulating peripheral blood monocytes after rosuvastatin treatment. The results of this study indicate that after 2 months of rosuvastatin therapy, the levels of PPAR- γ mRNA and activated PPAR- γ protein expression are significantly increased in monocytes. A previous work indicates that statins can activate PPAR- γ in monocytes and macrophages¹⁹. The experimental results demonstrated that in the *in vitro* human monocytes, rosuvastatin can induce PPAR- γ expression and activation, which is consistent with the previous study. Also, this effect is in

a dose-dependent manner. Based on the above findings, rosuvastatin may induce the differentiation of monocytes into anti-inflammatory M2 macrophages by activating PPAR- γ .

Since M1 and M2 macrophage markers can be found in atherosclerosis lesions, the concepts related to macrophage heterogeneity has entered the field of atherosclerosis research. Our work indicates that PPAR- γ expression and activation in monocytes can be induced by rosuvastatin *in vivo* and *in vitro*, promoting further exploration on the effect of rosuvastatin in the differentiation of monocytes into M2 macrophages. As the assumption, under the stimulation by IL-4, rosuvastatin can induce the differentiation of monocytes into M2 macrophages, and M2 macrophages activated by rosuvastatin can inhibit M1 macrophage inflammatory effect and play an anti-inflammatory effect on M1 macrophages via paracrine. This effect can be amplified by PPAR- γ agonist and can also be completely inhibited by PPAR- γ antagonist T0070907, suggesting that rosuvastatin exerts an anti-inflammatory effect in macrophages mainly through the activation of PPAR- γ . Most importantly,

these results suggest the existence of a molecular pathway, and statins can play an anti-inflammatory role in vasculature, inhibit platelet deposition and establish stable atherosclerotic plaques via this way.

As discussed above, the differentiation of monocytes into M2 macrophages promoted by rosuvastatin involves the activation of PPAR- γ . In fact, PPAR- γ can be affected by the negative regulation of phosphorylated mitogen-activated protein kinase (MAPK)²⁰. However, in human monocytes and macrophages, the phosphorylation of PPAR- γ serine residues cannot be induced by rosuvastatin²¹. Therefore, PPAR- γ activation induced by rosuvastatin cannot be achieved through inhibiting phosphorylation of MAPK serine residues. The previous report shows that in murine macrophages, statins can induce PPAR- γ activation through activating ERK1/2 and p38 MAPK²², but in the human monocytes used in this study, rosuvastatin can significantly induce p38 MAPK phosphorylation, which is not obvious on the ERK1/2. Meanwhile, when p38 MAPK inhibitor SB203580 is added, PPAR- γ expression and activation induced by rosuvastatin are inhibited, which are not distinctly affected by the specific inhibitor PD98059 of MAPK/ERK kinase. Notably, the detection results of CD206 and CD163 mRNA and protein levels also revealed that the differentiation of monocytes into M2 macrophages induced by rosuvastatin can be blocked by SB203580, not PD98059. At present, the mechanisms behind these phenomena are not yet fully understood.

Conclusions

For the first time we have found a large number of expressions of M2 markers in human circulating peripheral blood monocytes after rosuvastatin therapy. In addition, both *in vivo* and *in vitro* experiments have demonstrated that rosuvastatin can also induce the expression and activation of PPAR- γ in human monocytes, resulting in the differentiation of monocytes into M2 macrophages. Our research also confirmed that PPAR- γ activation mediated by rosuvastatin and differentiation of monocytes tending into M2 macrophages is achieved via p38 MAPK, not ERK1/2. This study laid a solid biological foundation for further exploring the mechanism of rosuvastatin.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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