Original Article

Effect of Sitagliptin on the anti-atherogenic M2 macrophage polarization

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Abstract: Dipeptidyl peptidase 4 (DPP-4) inhibitors are a new class of anti-hyperglycaemic drugs that can improve blood glucose by reducing inactivation of incretins. Studies have shown that these drugs can exert anti-atherosclerotic and anti-inflammatory effects in mice. However, the roles of DPP-4 inhibitors in polarization of macrophages have not been well studied. Therefore, we investigated the effects of DPP-4 inhibitor Sitagliptin on macrophage polarization. Monocyte-derived macrophages were established from human monocytic leukaemia THP-1 cells treated with phorbol myristate acetate (PMA). Lipopolysaccharide (LPS, 20 ng/ml) stimulation was used to initiate inflammatory conditions. We found that Sitagliptin could induce macrophage polarization towards M2 cells through activating STAT6. Sitagliptin treatment significantly up-regulated the specific markers of M2 macrophage expression, including the scavenger receptor CD163 and the macrophage mannose receptor CD206. Furthermore, pre-treatment of macrophages with Sitagliptin for 24 h also resulted in the up-regulation of mRNA levels of IL-10, TGF-β and Arg-1, which are markers related to M2 macrophages, while the mRNA levels of TNF-α, IL-6 and iNOS were decreased. An increase in the levels of the anti-inflammatory cytokines, IL-10 and TGF-β was also observed. Moreover, a reduction in oxidized low density lipoprotein (ox-LDL)-induced lipid accumulation was observed in Sitagliptin-treated M2 macrophages confirming the anti-atherogenic effect of DPP-4 inhibitor. In conclusion, the results of present study showed that Sitagliptin-induced M2 polarization of macrophages could be mediated by STAT6 signalling, which facilitated its anti-inflammatory and anti-atherogenic properties.

Keywords: DPP-4, atherosclerosis, macrophage, anti-inflammation, STAT6

Introduction

Cardiovascular events based on atherosclerosis are the leading cause of mortality in patients with type 2 diabetes [1]. Atherosclerosis is now considered a chronic inflammatory disease. A large amount of evidence suggests that inflammation is associated with all phases of the atherosclerotic process [2, 3]. In addition, among the numerous cell types involved in local inflammation, macrophages are fundamental contributors. Macrophages (MΦ) are broadly classified into classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages). M1 macrophage can produce pro-inflammatory cytokines, including tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), while M2 cells can release anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) [4]. Thus, macrophages are the major inflammatory cells in atherogenesis and play important roles in the progression of lesions. Since it has been demonstrated that MΦ subsets display some degree of plasticity and heterogeneity in atherosclerotic lesions, recently the procedure of MΦ polarization during atherogenesis has become a subject of interest [5]. Therefore, an appreciation of the anti-atherosclerotic and anti-inflammatory effects of anti-hyperglycaemic agents has burgeoned, especially study focused on the stage of macrophage polarization.

Dipeptidyl peptidase 4 (DPP-4), also known as CD26, is an enzyme that inactivates glucagon-like peptide 1 (GLP-1). GLP-1 is an incretin hormone that improves blood glucose by increasing insulin secretion and suppressing glucagon secretion in the pancreas [6, 7]. DPP-4 inhibi-
Sitagliptin and M2 macrophage differentiatators are a new class of oral hypoglycaemic agents that can suppress the inactivation and degradation of GLP-1 [8]. Recently, some studies have reported that Sitagliptin, a DPP-4 inhibitor, plays a role in the prevention of inflammation and atherosclerosis [9-11]. Several studies have verified that DPP-4 inhibitors can not only suppress the inflammatory reactions of monocytes and proliferation of vascular smooth muscle cells, but also reduce leukocyte-endothelial cell interactions and the area of the atherosclerotic lesion. Furthermore, they can improve endothelial function [10, 12, 13]. However, the precise mechanism underlying the effects of Sitagliptin on macrophage differentiation has not been well studied [14, 15].

In the present study, we explored the effects and mechanisms of Sitagliptin on M2 macrophage polarization and inflammatory cytokine at gene and protein levels in vitro by using flow cytometry, ELISA, Western blot analysis and real time PCR. We also further studied mechanism of its inhibition on the formation of foam cells.

Materials and methods

Chemicals

Phorbol-12-myristate-13-acetate (PMA) and lipopolysaccharides (LPS) were purchased from Sigma Chemical Company (St. Louis, MO, USA). AS1517499, a STAT6 inhibitor, was obtained from Merck Millipore (Billerica, MA, USA). All of the cell culture products were obtained from Hyclone Laboratories, Inc. (HyClone, UT, USA).

Cultivation of monocyte cells

Human THP-1 cells (ATCC, Rockefeller, MD, USA) were cultured in RPMI1640 medium (HyClone, UT, USA) containing 10% fetal bovine serum (Australia origin (FBS, Excell Bio, Shanghai, China)) and 20 µg/ml penicillin-streptomycin solution at 37°C with 5% CO₂. Prior to experimentation, THP-1 cells were seeded at a density of 5 x 10⁵/ml per plate in 6-well cell culture plates (Corning, NY, USA) and treated with 10 ng/ml PMA for 24 h to generate macrophage-like cells. The cells were then treated with LPS (20 ng/ml) to stimulate the expression of inflammatory signals. Sitagliptin (Santa Cruz, CA, USA) was dissolved in sterile deionized water and stored at 4°C in the dark. Sitagliptin was added 1 h before LPS stimulation, with or without a pre-treatment of the STAT6 inhibitor AS1517499 100 nM for 0.5 hour [16]. Samples for protein analysis were collected 6 h after treatment with LPS, and samples for mRNA analysis were obtained after a 24-hour treatment with LPS.

Flow cytometry analysis

Cells were treated for 24 h with LPS and then washed twice with PBS. The expression of the cell surface antigens CD206 and CD163 was determined by flow cytometry. PE mouse anti-human CD206 and PE mouse anti-human CD163 were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). The stained cells were assayed with a flow cytometer (BD FACS Calibur; Becton, Dickinson and Company, Franklin Lakes, NJ) and analyzed with BD Cell Quest software (Becton, Dickinson and Company, Franklin Lakes, NJ).

ELISA assay

The levels of TNF-α, IL-6, IL-10 and TGF-β were measured using ELISA kits (eBioscience San Diego, CA, USA). The procedures were performed according to the manufacturer’s instructions. The reactions were stopped with 2 M H₂SO₄, and the absorbance was read using an enzyme-labelled instrument (Bio-Rad, CA, USA) at 450 nm. The concentration of each cytokines was calculated based on the standard curves and absorbance value.

Quantitative RT-PCR analysis

Total RNA was extracted from THP-1 cells using the TRIzol reagent (Invitrogen, USA) to identify the expressions of M1 or M2 polarization marker proteins in macrophages. The cDNA was synthesized using 1 µg RNA with PrimeScriptRT master mix (Perfect Real Time) (TaKaRa Bio Inc. Japan). Quantification of the mRNA was performed in triplicate using a LightCycler480 II (Roche, Applied Science) with SYBR Premix Ex Taq™ (Perfect Real Time) (TaKaRa Bio Inc. Japan). The results were analyzed with Roche LightCycler 2.0 software and normalized according to the expression levels of GAPDH RNA. Primers were used to amplify the following genes were found in Primer Bank:

TNF-α (5'-AGACCAAGGTCACCTCCTCT-3'; 5'-TCGGGCGATTGATCTCA-3'); IL-6 (5'-TGGCTGA-
AAAAGATGGATGCT-3', 5'-TCTGCACAGCTCTGGCTTGT-3'); Inos (5'-TCATCCGCTATGCTGGCTAC-3', 5'-CCCGAAACCACTCGTATTTGG-3'); IL-10 (5'-CAGCTCAGCACTGCTCTGTTG-3', 5'-TTCACTCTGCGAAGGCATCTC-3'); TGF-β (5'-CAGCAACAATTCC-TGGCGATACCT-3', 5'-CGCTAAGGCGAAAGCCCTCAAT-3'); Arg-1 (5'-TGGACAGACTAGGAATTGGCA-3', 5'-CCAGTCCGTCAACATCAAAAC-3').

Western blot analysis

After designated treatment, macrophages were harvested and suspended in RIPA lysis buffer (Beyotime, Jiangsu, China). The protein concentrations were determined using a BCA protein assay (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Proteins (40-55 μg) were resolved by SDS-PAGE and electrophoretically transferred to PVDF membranes, which were blocked with 5% skim milk in TBST (TBS containing 0.5% Tween-20) for 1.5-2.5 h on a shaking bed at room temperature. The membranes were then washed 3 times with TBST for 3-5 min each before incubated with following special diluted primary antibodies: STAT6 (1:1000, CST), p-STAT6 (1:1000, CST), STAT3 (1:1000, CST) and p-STAT3 (1:1000, CST), in TBS containing 5% BSA and 0.1% Tween-20 at 4°C overnight. After washing with TBST, the membranes were incubated with fluorescently labelled goat anti-rabbit or anti-mouse IgGs (1:10,000, Rockland) on a shaking bed at room temperature for 1 h. Positive bands were detected using an Odyssey Infrared Imaging System (Gene Company). The results were analyzed with ImageLab software (Bio-Rad, CA, USA).

Foam cell formation and Oil Red O staining

THP-1 cells (5 × 10⁵/ml per plate) were seeded in 6-well plates and cultured overnight. The
Sitagliptin and M2 macrophage
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Figure 2. Surface antigen expression of CD206 (A) and CD163 (B) after macrophages were stimulated with LPS (20 ng/ml) for 24 h. The expression levels of CD206 and CD163 increased significantly in response to pre-treatment with Sitagliptin, and this increase was inhibited by AS1517499. The data is presented as the mean ± SD (n = 3). Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, ns indicates no statistical significance.

Figure 3. mRNA levels of cytokines and enzymes produced by M1/M2 macrophages after a 6-hour pretreatment with 100 µM Sitagliptin followed by a 24-hour stimulation with LPS. Sitagliptin up-regulated the mRNA levels of the M2 macrophage-specific markers, IL-10 (D), TGF-β (E) and Arg-1 (F), as compared with the group treated with LPS alone, and this up-regulation could be inhibited by a STAT6 inhibitor. Sitagliptin down-regulated the mRNA levels of TNF-α (A), IL-6 (B) and iNOS (C), as compared with the group treated with LPS alone. The data is presented as the mean ± SD (n = 3). Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, ns indicates no statistical significance.

Cells were stimulated with LPS for 24 hours after pre-treatment with Sitagliptin in the absence or presence of the STAT6 inhibitor AS1517499 for 0.5 hour. Next, the cells were incubated with ox-LDL (50 mg/L) for 48 h. After 3 washes with PBS, the cells were fixed with 4% formaldehyde for 30 min at room temperature. After washing with distilled water, the cell lipids were stained with 3 mg/ml Oil Red O in 60% isopropanol for 10 min. Counterstaining was then performed with haematoxylin for 10 min, followed by rinsing with PBS. Images of the stained cells were acquired by microscopy (Life Inc., USA) and the numbers of total cells and foam cells were counted.

Statistical analyses

The results are expressed as the mean ± SD. One-away ANOVA and Newman-Keuls-Student’s t test (GraphPad Prism version 6.0) were used to analyze statistically significant differences among the groups. P < 0.05 was considered statistically significant.

Results

**Sitagliptin enhances the activation of STAT6 in monocyte-derived macrophages**

After pre-treatment with Sitagliptin, THP-1 cells were then stimulated with LPS (20 ng/ml). Proteins were then harvested 6 hours after stimulation. Both STAT3 and STAT6 play important roles in regulating macrophage differentiation towards the M2 phenotype [4]. To identify the effects and underlying mechanisms of DPP-4 inhibitors on macrophage activation, we first measured the effects of Sitagliptin on the activation of STAT3 and STAT6. Our data showed...
Sitagliptin and M2 macrophage

Figure 4. Effects of Sitagliptin on the secretions of pro-inflammatory and anti-inflammatory cytokines of macrophages. Sitagliptin pre-treatment resulted in decreased secretion of TNF-α (A) and IL-6 (B) and increased secretion of IL-10 (C) and TGF-β (D) compared with LPS alone. The increase in IL-10 (C) and TGF-β (D), but not the decrease could be inhibited by STAT6 inhibitor. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, ns indicates no statistical significance.

That compared with the group treated with LPS alone, treatment with Sitagliptin dose-dependently promoted STAT6 phosphorylation (P < 0.05, Figure 1D), but had no effect on STAT3 activation (Figure 1A). Since STAT6 activation is a key event in macrophage activation towards the M2 phenotype, it has been suggested that DPP-4 inhibitors may induce M2 activation of macrophages.

Sitagliptin induces M2 phenotype via activation of Stat-6

Compared with the group treated with LPS alone, the expression levels of the M2 phenotype macrophage makers, CD206 and CD163, were both significantly increased in the group that was pre-treated with Sitagliptin (P < 0.05, Figure 2A3, 2A4, 2B3, 2B4). This result suggested that Sitagliptin induced an increase in the percentage of M2 cells. To identify whether this effect occurred via STAT6 signalling, we exposed THP-1 cells to AS1517499, an STAT6 inhibitor, for 0.5 h prior to treating them with DPP-4 inhibitor. In the group that was pre-treat-
ed by Sitagliptin and AS1517499, CD206 and CD163 expression clearly decreased (P < 0.05, Figure 2A6, 2B6). This finding indicated that Sitagliptin induced macrophage activation towards the M2 phenotype via phosphorylation of Stat-6 signalling.

Sitagliptin up-regulates the expression of gene profiles typical of M2 cell phenotype

To further examine the effect of Sitagliptin on macrophage polarization in an inflammatory microenvironment, we next analyzed the gene expression of typical markers of classically activated (M1) macrophages and alternatively activated (M2) macrophages. Stimulation with LPS resulted in a reduction in arginase-1 (Arg-1) compared with the control group, and this reduction was inhibited by pre-treatment with Sitagliptin (Figure 3F). The expressions of other M2 markers such as IL-10 and TGF-β, also increased after pre-treatment by Sitagliptin (P < 0.05, Figure 3D, 3E). In the present study, we confirmed that Sitagliptin inhibits LPS induced M1 polarization in monocyte-derived macrophages. In addition to suppressing inducible nitric oxide synthase (iNOS) expression at the mRNA levels, treatment with Sitagliptin resulted in a reduction in TNF-α (P < 0.05, Figure 3C, 3A). A decreasing trend was also observed for the mRNA level of IL-6 (P < 0.05), compared with the group that was stimulated with LPS alone (Figure 3B). These effects of Sitagliptin on the mRNA levels in M2 but not M1 cells could be inhibited by the STAT6 inhibitor.

Effects of DPP-4 inhibitors on pro-inflammatory and anti-inflammatory cytokine activity

It is known that pro-inflammatory and anti-inflammatory cytokine activity produced by M1 and M2 macrophages, provides a potential link between inflammation and insulin resistance [17]. To confirm that DPP-4 inhibitors could induce and inhibit the release of anti-inflamma-
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Figure 5. Regulation of lipid accumulation in macrophages by Sitagliptin. Oil Red O staining of ox-LDL incubated macrophages pre-treated with LPS alone (B), LPS together with Sitagliptin (C), or LPS together with Sitagliptin and STAT6 inhibitor, AS1517499 (D) and control group (A). In Sitagliptin-treated M2 macrophages, a reduction of LPS-induced lipid accumulation was observed. This decrease was recovered in the presence of STAT6 inhibitor. The ns indicates no statistical significance.

Sitagliptin reduces foam cell formation in LPS-treated macrophages

Although a reduction in atherosclerotic plaques by Sitagliptin has been well established in Apoe⁻/⁻ mice [9-11], whether the Sitagliptin-induced polarization of macrophages the M2 phenotype has beneficial effects on foam cell formation during plaque progression remains unclear. Therefore, to clarify whether Sitagliptin-induced alternative activation of macrophages could have an anti-atherosclerotic effect, the formation of foam cells was assessed in LPS-treated macrophages with or without Sitagliptin pre-treatment. In the group that was pre-treated with Sitagliptin, a reduction of LPS-induced lipid accumulation was observed, in comparison to the group that was stimulated by LPS alone (Figure 5B, 5C). To evaluate the role of STAT6 signalling in Sitagliptin-induced improvements in lipid accumulation, the STAT6 inhibitor, AS1517499, was used. Pre-treatment with Sitagliptin and AS1517499 reversed the reduction in foam cells induced by Sitagliptin (Figure
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This result strongly suggests that p-STAT6 activation is a key mechanism underlying the anti-atherogenic effects of Sitagliptin-induced M2 macrophage polarization.

Discussion

Diabetes, one of the major risk factors for atherosclerosis, influences the development and progression of atherosclerosis by creating a pro-inflammatory environment [18]. Clinical trials have demonstrated that the main causes of death and disability in patients with diabetes are vascular complications and events. It’s well known that atherosclerosis is the pathologic basis of vascular complications in these patients [1]. Inflammation appears to be responsible for the pathogenesis of all stages of atherogenesis, and it involves numerous cell types. Among these cell types, macrophages, as a consequence of their pro- and anti-inflammatory functions, can influence atheroma evolution and outcome, and they are fundamental contributors to the atherosclerotic process [4]. Therefore, investigations of the anti-atherosclerotic and anti-inflammatory effects are associated with anti-diabetic agent-induced macrophage polarization has become a new research focus. The new class of anti-hyperglycaemic drugs, known as DPP-4 inhibitors, have demonstrated promising results in this area of study.

Recently, Junichi Matsubara have showed that Sitagliptin significantly improved endothelial cell functions and reduced the formation of atherosclerotic lesions in Apoe⁻/⁻ mice [10]. Nasib Ervinna showed that Anagliptin suppressed monocyte inflammatory reactions and vascular smooth muscle proliferation [12]. Zubair Shah demonstrated that Alogliptin reduced inflammation by inhibiting monocyte activation/chemotaxis [13]. Although C. Brenner lately found that Sitagliptin primed monocytes into M2 macrophages, the mechanism has not been well studied [15]. The anti-inflammatory and anti-atherosclerotic effects of DPP-4 inhibitors have been described in some studies, however, most of underlying mechanisms are dependent on incretins. Since inflammation-driven atherosclerosis is firmly associated with endothelial cells, macrophages and smooth muscles cells, the effects of DPP-4 inhibitors on inflammation, especially on macrophage polarization, may be an interesting topic for further investigation.

In the present study, we further investigated the effects and underlying mechanisms of Sitagliptin on macrophage phenotypes, especially on M2 polarization in THP-1 macrophages. STAT6 is one of the primary signalling pathways involved in macrophage polarization towards the M2 phenotype [4, 19]. However, the relationship between DPP-4 inhibitor and STAT6 signalling pathway has not been described. Our results showed that pre-treatment with Sitagliptin resulted in the up-regulation of the typical M2 macrophage markers, CD206 and CD163, and this effect could be inhibited by a STAT6 inhibitor. This finding suggests that phosphorylation of STAT6 induced by Sitagliptin is the key mechanism underlying the polarization of the M2 phenotype. In addition, STAT6 but not STAT3 pathway activation was required for Sitagliptin-induced M2 polarization of macrophages. Furthermore, M2 polarization resulted in anti-atherogenic macrophages.

Atherosclerosis is a type of inflammatory disease. Our data showed that Sitagliptin induced not only an increase in the mRNA level of the M2 macrophage markers, TGF-β, IL-10 and Arg1 , but also in the activities of TGF-β and IL-10, which are anti-inflammatory cytokines produced by M2 cells. These increases could be inhibited by AS1517499, a STAT6 inhibitor. Sitagliptin also decreased the mRNA level of the markers of M1 macrophage, TNF-α, IL-6 and iNOS, and it suppressed the activities of TNF-α and IL-6. These reductions could be partially inhibited by a STAT6 inhibitor. In summary, Sitagliptin provides anti-inflammatory effects via STAT6 signaling. Our data suggests that in addition to their anti-diabetic effects, DPP-4 inhibitors also have anti-inflammatory and anti-atherosclerosis functions, and that these effects are likely to be dependent on STAT signalling.

Our study showed that LPS down-regulated markers of M2 macrophage expression, which is similar to other previous research [20]. However, we found that LPS could weakly activated STAT3 signalling, and this should have resulted in an up-regulation of M2 makers. We speculate that LPS may affect other signalling pathways that inhibit markers of M2 macrophage expression, and the promotion caused by LPS on STAT3 signalling cannot confront the inhibition by those signalling pathways.
This is the first study to demonstrate a relationship between Sitagliptin and STAT6 signalling. We focused on the atheroprotective effects of DPP-4 inhibitors that occurred independently of incretins. Our results demonstrating the anti-atherogenic effects of Sitagliptin are consistent with previous research reported by other groups. In addition, we investigated the mechanisms underlying some of these effects. A limitation of our study is that we only examined the relationship between DPP-4 inhibitors and STAT6 signalling in cells but not in vivo. In addition, we were unable to provide direct evidence of the relationship between CD26 and STAT6. Because our research focused on the effects of DPP-4 inhibitor on M2 macrophage polarization, we were unable to verify the mechanism(s) by which DPP-4 inhibitors either reduce mRNA levels of M1 cell markers or suppressing pro-inflammatory cytokines activities. These outstanding questions will be investigated in future studies.

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Disclosure of conflict of interest

None.

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