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Glucagon-like peptide-1 (GLP-1) induces M2 polarization of human macrophages via STAT3 activation

Daisuke Shiraishi [a,b,1], Yukio Fujiwara [a,1], Yoshihiro Komohara [a], Hiroshi Mizuta [b], Motohiro Takeya [a,*]

[a] Department of Cell Pathology, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Kumamoto, Japan
[b] Department of Orthopaedic Surgery, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Kumamoto, Japan

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ABSTRACT

It is known that glucagon-like peptide-1 (GLP-1) is a hormone secreted postprandially from the L-cells of the small intestine and regulates glucose homeostasis. GLP-1 is now used for the treatment of diabetes because of its beneficial role against insulin resistance. The GLP-1 receptor (GLP-1R) is expressed on many cell types, including macrophages, and GLP-1 suppresses the development of atherosclerosis by inhibiting macrophage function. However, there have so far been few studies that have investigated the significance of GLP-1/GLP-1R signaling in macrophage activation. In the present study, we examined the effect of GLP-1 and exenatide, a GLP-1R agonist, on human monocyte-derived macrophage (HMDM) activation. We found that GLP-1 induced signal transducer and activator of transcription 3 (STAT3) activation. Silencing of GLP-1R suppressed the GLP-1-induced STAT3 activation. In addition, alternatively activated (M2) macrophage-related molecules, such as IL-10, CD163, and CD204 in HMDM, were significantly upregulated by GLP-1. Furthermore, the co-culture of 3T3-L1 adipocytes with GLP-1-treated RAW 264.7 macrophages increased the secretion of adiponectin compared to co-culture of the 3T3-L1 adipocytes with untreated RAW 264.7 macrophages. Our results demonstrate that GLP-1 induces macrophage polarization toward the M2 phenotype, which may contribute to the protective effects of GLP-1 against diabetes and cardiovascular diseases.

1. Introduction

The incretin hormone, glucagon-like peptide 1 (GLP-1), is released from intestinal L cells following meal ingestion. GLP-1 stimulates insulin secretion and decreases glucagon secretion, reduces gastric emptying, and promotes satiety in a glucose-dependent manner [1]. The physiological relevance and pharmacology of GLP-1 have been researched extensively, with a major focus on its incretin actions, and its application in the treatment of type-2 diabetes. However, the administration of GLP-1 is not effective as a treatment for diabetes, because the protein is rapidly degraded by dipeptidyl peptidase-4 (DPP-4). Thus, GLP-1 receptor agonists that are resistant to DPP-4 and DPP-4 inhibitors are currently being used for the treatment of type 2 diabetes [2]. In addition, GLP-1 receptors are abundantly expressed in many cell types other than pancreatic islet cells, including gastrointestinal cells, and neural cells [1]. It was recently reported that GLP-1 receptor agonists (exenatide or exendin-4) reduced monocyte/macrophage accumulation in the arterial wall by inhibiting the inflammatory response in macrophages and attenuated atherosclerosis [2]. Therefore GLP-1 has been suggested to be involved in macrophage activation and polarization.

Macrophages are broadly classified into classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages), according to their roles. M1 macrophages are potent effecter cells that kill microorganisms and produce primarily proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-6, and IL-12 [3]. In contrast, M2 macrophages reduce these inflammatory and adaptive Th1 responses by producing anti-inflammatory factors (IL-10, TGF-β and IL-1 receptor antagonist), and promote angiogenesis, tissue remodeling, and repair [3]. Macrophages are plastic cells, because they can switch from an activated M1 state back to M2, and vice versa, upon the induction of specific signals [3]. The signal transducer and activator of transcription 3 (STAT3) signaling in macrophages is well known to be involved in the regulation of immune responses in murine models [4,5], and STAT3 activation is essential for macrophage differentiation toward the M2 phenotype [6].

It has also been reported that adipose tissue macrophages (ATMs) from lean mice express many genes characteristic of M2 macrophages, which protect adipocytes from inflammation, whereas diet-induced obesity led to a shift in the activation state to an M1 pro-inflammatory state that contributes to insulin resistance [7–9], thus suggesting that macrophage activation may thus play an important role in the functions of adipocytes.

* Corresponding author. Fax: +81 96 373 5096.
E-mail address: takeya@kumamoto-u.ac.jp (M. Takeya).
[1] These authors contributed equally to this work.

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To examine the effect of GLP-1 on macrophage activation and function, we performed an *in vitro* study using human macrophages. In the present study, we mainly focused on STAT3 signaling in macrophages, and we demonstrated that GLP-1 induced macrophage differentiation into the M2 phenotype via STAT3 activation. In addition, the effect of GLP-1 on cell–cell interactions between macrophages and adipocytes was also investigated, and we found that GLP-1-stimulated macrophages had enhanced adiponectin secretion from adipocytes. These findings suggest a new mechanism involved in the protective role of GLP-1R agonists and DPP-4 inhibitors against diabetes and cardiovascular disease.

2. Materials and methods

2.1. Chemicals

Human GLP-1 was purchased from PeproTech (NJ, USA). Exenatide was purchased from GenScript (NJ, USA). All other chemicals were of the best grade available from commercial sources.

2.2. Cells and cell culture conditions

Peripheral blood mononuclear cells were obtained from healthy volunteer donors. Informed written consent was obtained from all healthy donors. $CD14^+$ monocytes were purified from peripheral blood mononuclear cells by positive selection via magnetic-activated cell sorting technology (Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytes were cultured in DMEM supplemented with 2% FBS and 5 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF, Wako, Tokyo, Japan) for 5 days in order to differentiate into macrophages.

RAW 264.7 cells and mouse 3T3-L1 pre-adipocytes were purchased from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.1 mg/mL sodium pyruvate. To set up the 3T3-L1 pre-adipocytes for differentiation and experimentation, the cells were exposed to differentiation medium, which was the growth medium additionally supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone, and 1 μg/mL insulin, for 7 days.

2.3. Determination of the effect of GLP-1 on CD163 expression

The purified monocytes (5×10^4 cells per well of a 96-well plate) were incubated with GLP-1 (10 nM) for 48 h, and CD163 expression was evaluated by cell enzyme-linked immunosorbent assay (Cell-ELISA) as described previously [10]. Briefly, following the cells were incubated with anti-CD163 antibody, AM-3K, (2 μg/ml), and they were reacted with anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP). Then cells were incubated in TMB solution (Moss INC., Pasadena, MD, USA).

2.4. Determination of the effect of GLP-1 on IL-10, IL-12 and TNF-α secretion

The monocytes (5×10^4 cells per well of 96-well plate) were stimulated with LPS (100 ng/ml) for 24 h after incubation with GLP-1(10 nM) for 48 h, followed by the determination of IL-10, IL-12 and TNF-α secretion by means of an ELISA kit (eBioscience, San Diego, CA, USA).

2.5. Western blot analysis

STAT3 activation was determined by measuring the increased expression of the phosphorylated STAT3 by Western blot analysis, as described previously [11,12]. CD204 expression was also evaluated by Western blot analysis as described previously [13]. Briefly, macrophages were solubilized with Triton X-100, and the protein concentration was determined using the BCA protein assay reagent, followed by pretreatment by boiling for 5 min in 2% SDS and 2-mercaptoethanol. The protein (10 μg) was run on a 10% SDS–polyacrylamide gel and transferred to a polyvinylidine fluoride (PVDF) transfer membrane (Millipore, Bedford, MA, USA). To detect STAT3, phosphorylated STAT3, and CD204, the membranes were exposed to anti-STAT3 antibody (sc-8019; Santa Cruz Biotech, Santa Cruz, CA, USA), anti-phosphorylated STAT3 antibody (D3A7, Cell Signaling), and anti-CD204 antibody (SRA-E5, Transgenic, Kumamoto, Japan) respectively [14,15]. These membranes were re-blotted with an anti-β-actin antibody as an internal calibration control.

2.6. Determination of the effect of GLP-1 on adiponectin secretion

RAW cells were pre-treated with/without GLP-1 (10 nM) for 24 h, then co-cultured with 3T3-L1 adipocytes for 24 h, followed by determination of the adiponectin in the culture supernatant by a Mouse Adiponectin secretion ELISA kit (CycLex Co., Nagano, Japan) and a Western blot analysis. Prior to assessing the protein content, the culture supernatant (10 μl) of 3T3-L1 adipocytes was diluted by adding PBS, followed by pretreatment by boiling for 5 min in 2% SDS and 2-mercaptoethanol. The samples were then run on a 20% SDS–polyacrylamide gel and transferred to a polyvinylidine fluoride (PVDF) transfer membrane (Millipore, Bedford, MA, USA). To detect adiponectin, the membranes were exposed to an anti-mouse adiponectin antibody (MAB3608; Chemicon international Inc.) and visualized using a HRP-conjugated anti-rabbit IgG antibody with the ECL Western blotting detection reagent (GE Healthcare Japan, Tokyo, Japan). The molecular size of adiponectin detected by this immunoblot analysis was approximately 30 kDa.

2.7. RT-PCR

Total RNA was extracted with RNA iso-Plus (TaKaRa Biotechnology, Japan). The reverse transcription (RT) reaction was carried out using a PrimeScript™ RT Reagent kit with gDNA Eraser (TaKaRa, Japan), and the following primers were used to amplify the target genes: GLP-1R: 5′-GAACCTGTTTGCATCCTTCATC-3′, 5′-TACTGCATGAGCAGAAACACC-3′; β-actin: 5′-CTGTGGCATCCACGAAACTAC-3′, 5′-CTGATCCACATCTGCTGGAAG-3′. The PCR was performed for 40 or 30 cycles, respectively, and each cycle included denaturation at 98 °C for 10 s, annealing and extension at 60 °C for 30 s, before a final extension at 72 °C for 1 min.

2.8. Small interfering RNA

To inhibit the GLP-1R in macrophages, cells were transfected with siRNA against human GLP-1R (sc-45760; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with a control siRNA (sc-44230; Santa Cruz), using Lipofectamine RNAi MAX (Invitrogen) as described previously. [16].

2.9. Statistics

All data are representative of two or three independent experiments. Data are expressed as means ± SD. Mann–Whitney's *U*-test

was used for two-group comparison. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of GLP-1 and exenatide on STAT3 activation in HMDM

To identify the effects of GLP-1 on macrophage activation, we first measured the effects of GLP-1 and exenatide, a GLP-1R agonist, on the activation of STAT3, which plays an important role in macrophage differentiation toward the M2 phenotype in human monocyte-derived macrophages (HMDM). We observed that both GLP-1 and exenatide induced STAT3 activation in a dose-dependent manner (Fig. 1). Since STAT3 activation is a key event for macrophage activation toward the M2 phenotype, it has been suggested that GLP-1 may induce the M2 activation of macrophages.

3.2. Effect of GLP-1 receptor signaling on STAT3 activation in HMDM

A RT-PCR analysis revealed that GLP-1R expression was detected in monocytes and HMDM (Fig. 2A). However, there has been little information published on the effect of GLP-1 signaling in macrophages. To clarify whether GLP-1R signaling is involved in STAT3 activation in HMDM, we examined the effects of GLP-1 on STAT3 activation under GLP-1R siRNA-mediated knockdown conditions. As shown in Fig. 2B, transfection of the HMDM with GLP-1R siRNA decreased the GLP-1 induced-STAT3 activation. Furthermore, a synergistic effect of GLP-1 and IL-10 on STAT3 activation was observed (Fig. 2C). These observations indicate that GLP-1/GLP-1R signaling induces STAT3 activation in HMDM.

3.3. Effects of GLP-1 on the macrophage activation

Because STAT3 activation is known to be associated with the anti-inflammatory response in macrophages, we next evaluated the effects of GLP-1 on the cytokine secretion (IL-10, IL-12 and TNF-α) of HMDM. GLP-1 increased IL-10 secretion, a M2 phenotype

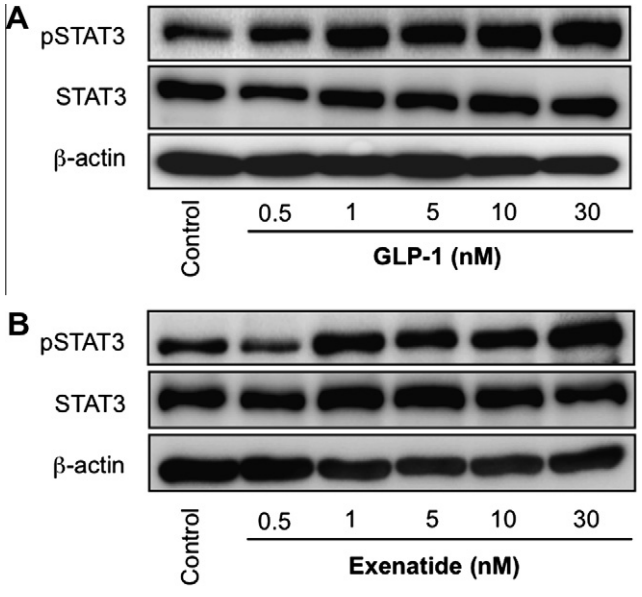

Fig. 1. Effects of GLP-1 and exenatide on STAT3 activation in macrophages. HMDM (4×10^5 cells per well of a 24 well plate) were incubated with the indicated concentrations of GLP-1 (A) and exenatide (B) for 24 h, followed by determination of the expression levels of STAT3, phosphorylated STAT3, and β-actin by a Western blot analysis as described in Section 2. All experiments were repeated three times with almost identical results.

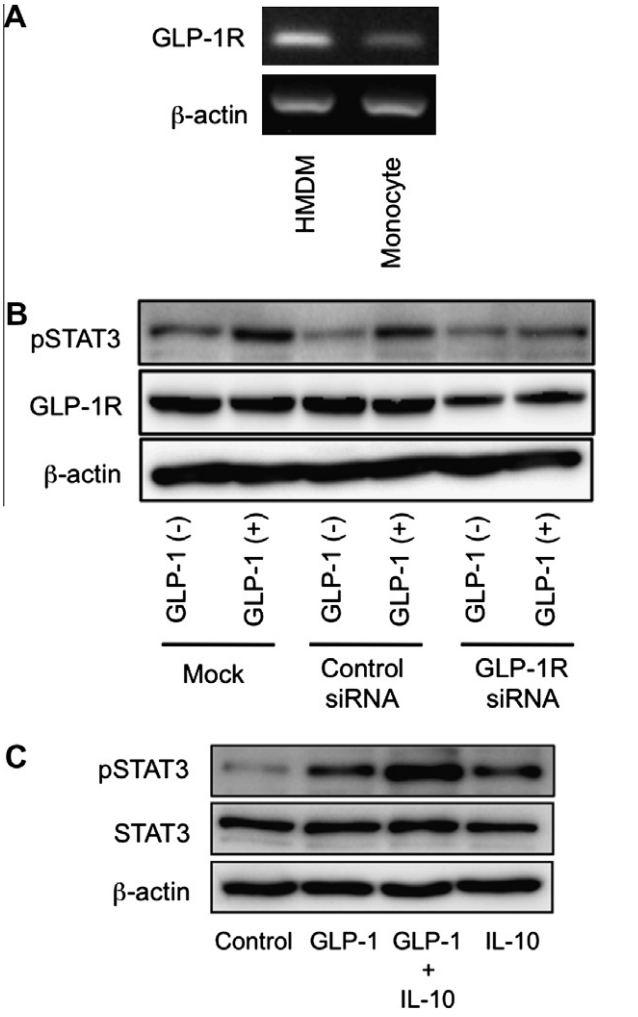

Fig. 2. Effects of GLP-1 receptor signaling on STAT3 activation in macrophages. The GLP-1R expression in human monocytes and HMDM was detected by RT-PCR as described in Section 2 (A). HMDM were incubated with GLP-1 (1 nM) for 24 h after treatment with control siRNA and GLP-1R siRNA, followed by determination of the expression levels of phosphorylated STAT3, GLP-1R and β-actin by a Western blot analysis as described in Section 2 (B). HMDM (4×10^5 cells per well of a 24 well plate) were incubated with GLP-1 (10 nM) and/or IL-10 (5 μg/mL) for 24 h, followed by determination of the expression of STAT3, phosphorylated STAT3, and β-actin by a Western blot analysis, as described in Section 2 (C). All experiments were repeated three times with almost identical results.

marker (Fig. 3A), whereas the IL-12 and TNF-α secretion, M1 phenotype markers, were not changed compared with the control cells (Fig. 3B and C). GLP-1 also increased the expression of both CD163 and CD204, M2 phenotype markers (Fig. 3D and E). These results demonstrate that GLP-1 induces macrophage activation toward the M2 phenotype.

3.4. GLP-1 stimulation enhances the adiponectin secretion upon co-culture of macrophages with adipocytes

It is known that adipose tissue macrophages (ATMs) produce proinflammatory cytokines that can block the action of insulin and inhibit adiponectin secretion from adipocytes in obese subjects, providing a potential link between inflammation and insulin resistance [7–9]. Therefore, it has been thought that GLP-1-stimulated macrophages may improve adiponectin secretion from adipocytes by their anti-inflammatory functions. To clarify the effect of GLP-1 on macrophage activation toward the M2 phenotype, we measured the adiponectin secretion from 3T3-L1 adipocytes

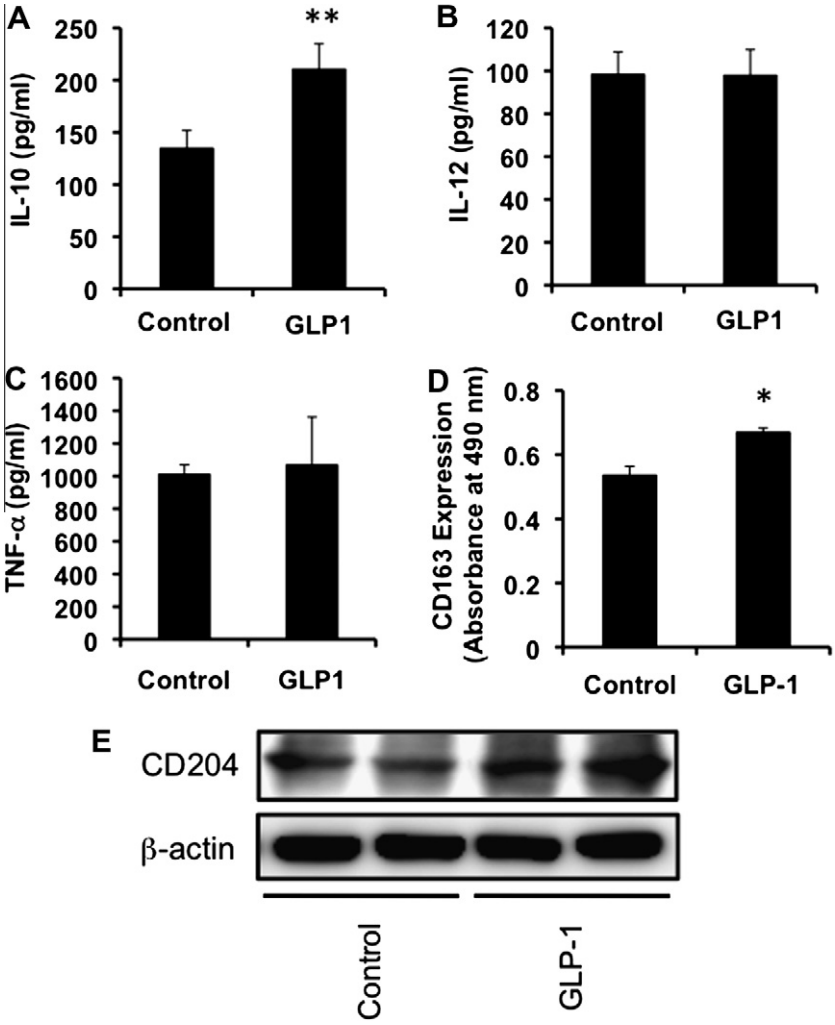

Fig. 3. Effects of GLP-1 on the macrophage activation. HMDM (5×10^4 cells per well of 96 well plates) were stimulated with LPS (100 ng/ml) for 24 h after incubation with GLP-1 (10 nM) for 48 h, followed by an analysis of the secretion of IL-10 (A), IL-12 (B), and TNF-α (C) by an ELISA as described in Section 2. HMDM (5×10^4 cells per well of a 96 well plate) were incubated with GLP-1 (10 nM) for 48 h, followed by a determination of the CD163 expression by a cell-ELISA as described in Section 2 (D). HMDM (4×10^5 cells per well of a 24 well plate) were incubated with the indicated concentrations of GLP-1 for 24 h, followed by determination of the expression of CD204 and β-actin by a Western blot analysis, as described in Section 2 (E). All experiments were repeated three times with almost identical results. The data are presented as means ± SD. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. control.

co-cultured with GLP-1-stimulated RAW cells (a mouse macrophage cell line). As shown in Fig. 4A, GLP-1 also induced STAT3 activation in RAW cells. The adiponectin secretion from 3T3-L1 adipocytes was significantly reduced by co-culture with RAW cells, whereas GLP-1-stimulated RAW cells did not influence the adiponectin secretion from 3T3-L1 adipocytes (Fig. 4B and C). These findings indicate that GLP-1 reverses the inhibitory effect of macrophages on adiponectin secretion.

4. Discussion

Macrophages are broadly classified into classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages), according to their roles. It is well known that M1 macrophage polarization is associated with inflammation and tissue destruction, whereas the M2 macrophage phenotype is associated with anti-inflammatory effects, wound repair and angiogenesis [17].

In the present study, we revealed that GLP-1 induces the activation of STAT3, which is essential for macrophage differentiation toward the M2 phenotype. GLP-1R is abundantly expressed on many cell types, and GLP-1/GLP-1R signaling is associated with PI3-K and cAMP [18]. However, there have been no studies that have investigated the association between GLP-1/GLP-1R signaling and STAT3 signaling. It is well known that STAT3 is one of main signaling molecules related to macrophage polarization toward the M2 phenotype [6,16,19]. Therefore, we hypothesized that GLP-1/GLP-1R signaling may induce macrophage polarization toward the M2 phenotype. In support of this hypothesis, GLP-1 and exenatide, a GLP-1R agonist, induced macrophage activation toward the M2 phenotype. Therefore, this study provides the first evidence that GLP-1/GLP-1R signaling correlates with STAT3 activation and macrophage polarization.

Several reports have demonstrated that obesity gives rise to a state of chronic and low-grade inflammation [7–9]. Under non-obese conditions, ATMs express markers of M2 macrophages, whereas obesity leads to a reduction of these markers and the induction of genes associated with M1 macrophages [7–9]. M1 macrophages then infiltrate the adipose tissue of obese subjects and induce adipose tissue inflammation and insulin resistance. Therefore, inducing a phenotypic switch from M1 to M2 by GLP-

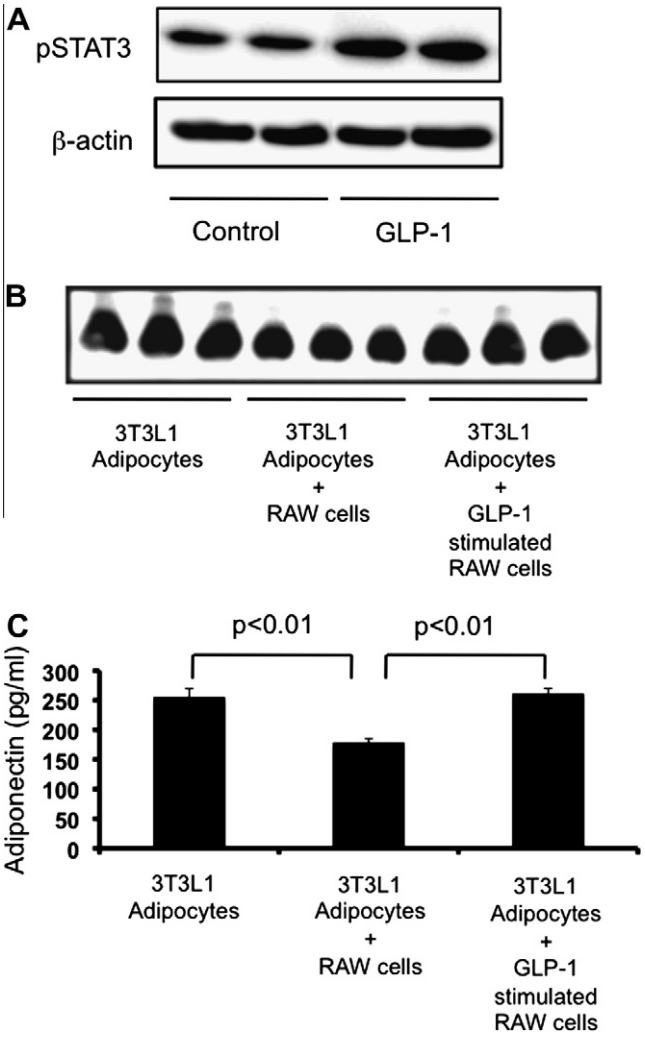

Fig. 4. Adiponectin secretion is increased when adipocytes are co-cultured with GLP-1-stimulated macrophages. RAW cells were incubated with the indicated concentrations of GLP-1 for 24 h, followed by determination of the expression levels of phosphorylated STAT3 and β-actin by a Western blot analysis as described in Section 2 (A). RAW cells were pre-treated with/without GLP-1 (10 nM) for 24 h, then co-cultured with 3T3-L1 adipocytes for 24 h, followed by determination of the adiponectin secretion in the supernatant by a Western blot analysis (B) and an ELISA (C) as described in Section 2. All experiments were repeated three times with almost identical results.

1 or a GLP-1R agonist is considered to be a new therapeutic strategy for type 2 diabetes [7,8]. It has been reported that GLP-1 and GLP-1R agonists directly promote the secretion of adiponectin, which is an important molecule related to insulin resistance and diabetes, from adipocytes [20,21]. In this study, we revealed that GLP-1 reversed the inhibitory effect of macrophages on adiponectin secretion from adipocytes (Fig. 4B and C), thus indicating that GLP-1 improves the functions of adipocytes by not only direct action on the adipocytes, but also indirectly by changing the macrophages toward the M2 phenotype (anti-inflammatory phenotype). Therefore, we suggest that the M2 polarization of ATMs by GLP-1/GLP-1R signaling is also involved in the therapeutic effects of GLP-1 agonists or DDP-4 inhibitors.

Based on the results of our present study, the promotion of macrophage polarization toward the M2 phenotype by GLP-1 might represent a new therapeutic strategy for diabetes, chronic inflammatory disease, cardiovascular diseases, and metabolic syndrome, through additional studies are required to clarify the detailed mechanism(s) underlying the effects of GLP-1 on macrophages.

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