A proline-type fullerene derivative inhibits adipogenesis by preventing PPARγ activation

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ABSTRACT

Obesity and its associated metabolic diseases represent some of the most rapidly expanding health issues worldwide, and, thus, the development of a novel chemical compound to suppress adipogenesis is strongly expected. We herein investigated the effects of water-soluble fullerene derivatives: a bis-malonic acid derivative and three types of proline-type fullerene derivatives, on adipogenesis using NIH-3T3 cells overexpressing PPARγ. One of the proline-type fullerene derivatives (P3) harboring three carboxy groups significantly inhibited lipid accumulation and the expression of adipocyte-specific genes, such as aP2, induced by the PPARγ agonist rosiglitazone. On the other hand, the bis-malonic acid derivative (M) and the 2 other proline-type fullerene derivatives (P1, P2), which have two carboxy groups, had no effect on PPARγ-mediated lipid accumulation or the expression of aP2. P3 fullerene also inhibited lipid accumulation induced by the combined stimulation with 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin in 3T3-L1 preadipocytes. During the differentiation of 3T3-L1 cells into adipocytes, P3 fullerene did not affect the expression of C/EBPδ, C/EBPβ, or PPARγ, but markedly inhibited that of aP2 mRNA. These results suggest that P3 fullerene exhibits anti-obesity activity by preventing the activation of PPARγ.

1. Introduction

Obesity is a major public health issue worldwide because it is a significant risk factor for a number of serious metabolic diseases such as heart disease, type 2 diabetes, atherosclerosis, and cancer [1,2]. An excess energy intake and lack of energy expenditure lead to adipogenesis and the accumulation of excessive amounts of lipids in adipocytes, which elevates triglyceride contents in plasma and tissues such as the liver and muscle, and ultimately results in pathological dysfunctions in these tissues [3,4]. Therefore, compounds that prevent the differentiation of adipocytes have potential as anti-obesity drugs.

The mechanisms underlying adipogenesis have been elucidated using murine 3T3-L1 preadipocytes. When treated with differentiation inducers termed MDI, which consist of 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin, 3T3-L1 preadipocytes differentiate into adipocytes, and this is regulated by the sequential expression of transcription factors such as CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPAR γ). The expression of C/EBPδ and C/EBPβ is induced in early adipocyte differentiation. C/EBPδ and C/EBPβ then cooperatively transactivate the expression of PPARγ, a master transcription factor for terminal adipocyte differentiation [5–7]. PPARγ induces the expression of adipogenic genes, including adipocyte Protein 2 (aP2), Glut4, and obesity genes, leading to the adipocyte phenotype [8–10].

Fullerene (C_{60}) is a spherical carbon molecule with a diameter of 0.7 nm, and is a new type of organic compound with a cage-like structure [11,12]. Since fullerene is poorly soluble in aqueous media, many water-soluble fullerene derivatives have been developed using modifications with hydrophilic groups in the C_{60} core. Several water-soluble fullerene derivatives have been reported to possess various biological and pharmacological properties [13,14]. A pyrrolidinium fullerene derivative has been shown

to induce apoptosis through the generation of reactive oxygen species (ROS), indicating their potential as anti-cancer drugs [15,16]. On the other hand, previous studies showed that a bis-malonic acid fullerene derivative exhibited potent anti-oxidant activity [17,18]. We recently demonstrated that a bis-malonic acid fullerene exhibited anti-inflammatory activity by inhibiting the activation of nuclear factor kappa B (NF-κB), which is a key transcription factor in mast cells (unpublished data). Furthermore, a proline-modified fullerene derivative was shown to exhibit antiviral activity by inhibiting human immunodeficiency virus (HIV)-reverse transcriptase and Hepatitis C virus RNA polymerase [19].

In the present study, we investigated the effects of fullerene derivatives on adipogenesis in order to clarify their anti-obesity activities. The results obtained demonstrated that a proline-type fullerene derivative, but not a bis-malonic acid fullerene derivative significantly inhibited lipid accumulation induced by the PPARγ agonist rosiglitazone in NIH-3T3 cells expressing PPARγ. The proline-type fullerene derivative also inhibited the differentiation of 3T3-L1 cells into adipocytes by preventing the activation of PPARγ. These results confirmed the potential of proline-type fullerene derivatives as novel anti-obesity drugs.

2. Materials and methods

2.1. Reagents and antibodies

A bis-malonic acid fullerene derivative and proline-type fullerene derivatives were synthesized as described previously [18,19]. Insulin, 3-isobutyl-1-methylxanthine (IBMX), and dexamethasone were purchased from CAYMAN CHEMICAL COMPANY (East Ellsworth Road Ann Arbor, MI, USA). Rosiglitazone and dimethyl sulfoxide (DMSO) were purchased from SIGMA ALDRICH (St. Louis, MO, USA) and Nacalai Tesque (Tokyo, Japan), respectively. Anti-C/EBPδ, anti-C/EBPβ, anti-PPARγ, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.2. Retroviral infection and cell cultures

The murine fibroblasts, NIH-3T3 cells were infected with an empty virus and retrovirus coding PPARγ-Flag as described previously [20]. Infected NIH-3T3 cells and murine 3T3-L1 preadipocytes were cultured at 37 °C with 5% CO_2-enriched air in DMEM (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) (BioWest), 100 units/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque). In order to induce differentiation, confluent 3T3-L1 cells were treated with 5 mM IBMX, 1 μM dexamethasone, and 10 μg/mL insulin (MDI). After 3 days, the medium was changed to DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10 μg mL insulin and cultured for another 2 days as previously described [21].

2.3. Oil-red O staining and quantification

3T3-L1 preadipocytes (3×10^4/well) were seeded on 96-well plates and adipocyte differentiation was induced for 6 days. the infected NIH-3T3 cells were seeded on 96-well plates and treated with rosiglitazone (1 μM) for 5 days. cells were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin for 30 min, then washed with 60% isopropanol. after air drying, 0.3% oil-red O solution was added to each well, and incubated at room temperature for 30 min. after the solution was removed, cells were washed with distilled water and air dried. Oil-red O in triglyceride droplets was extracted with 100% isopropanol and determined at OD_{490} for quantification as previously described [22].

2.4. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was prepared using Trizol (Life Technologies, Waltham, MA, USA). The reverse transcription reaction was performed using an oligo $(dT)_{20}$ primer and 2 μg of total RNA for first-strand cDNA synthesis, as described previously [21]. Quantitative real-time PCR was performed using an iCycler detection system (Bio-Rad, Berkeley, CA, USA). PCR was performed in 25 μL with SYBR Green Supermix, SsoFast EvaGreen (Bio-Rad). The PCR primer sequences used were as follows: C/EBPβ, 5′-ACAGCGACGAGTA-CAAGATCC-3′ (upstream) and 5′- GACAGTTGCTCCACCTTCTTCT-3′ (downstream); C/EBPδ, 5′-ATCGACTTCAGCGCCTACAT-3′ (upstream) and 5′-GCTTTGTGGTTGCTGTTGAA-3′ (downstream); PPARγ, 5′-AACTCTGGGAGATTCTCCTGTTGA-3′ (upstream) and 5′-TGGTAATTTCTTGTGAAGTGCTCATA-3′ (downstream); aP2, 5′-CATCAGCGTAAATGGGGATT-3′ (upstream) and 5′-TCGACTTTC-CATCCCACTTC-3′ (downstream); β-actin, 5′-TGTCCACCTTCCAG-CAGATGT-3′ (upstream) and 5′-AGCTCAGTAACAGTCCGCCTAGA-3′ (downstream).

2.5. Immunoblotting

Cell lysates were prepared with Nonidet P-40 lysis buffer (50 mM Tris–HCl, pH 7.4, 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 20 mM NaF, and 0.2 mM Na_3VO_4) supplemented with protease inhibitors. Proteins were denatured by adding Laemmli's sample buffer, and then resolved on 10% SDS-PAGE and transferred to PVDF membranes. Immunoblotting was performed as previously described [20].

2.6. Statistics

The significance of differences in Oil-red O staining and mRNA expression levels was determined using the Student's t-test with a confidence level of 95% ($P < 0.05$).

3. Results

3.1. Rosiglitazone induced the accumulation of lipid droplets and expression of aP2 in NIH-3T3 cells expressing PPARγ

The murine fibroblasts, NIH-3T3 cells were infected with a retrovirus harboring the cDNA of PPARγ-Flag, which is a master regulator for adipocyte differentiation (Fig. 1A). We determined whether these cells differentiated into adipocyte-like cells using the PPARγ agonist, rosiglitazone. NIH-3T3 cells expressing PPARγ were treated with rosiglitazone for 5 days, adipogenesis was assessed by Oil-Red O staining of lipid droplets, and lipid contents were quantified by measuring absorbance at 490 nm. The enforced expression of PPARγ induced lipid accumulation slightly more in NIH-3T3 cells than in cells infected with the empty virus (pBabe-Puro). Rosiglitazone markedly induced lipid accumulation in NIH-3T3 cells expressing PPARγ (Fig. 1B and C). Furthermore, whereas the expression of PPARγ slightly induced the expression of aP2 mRNA, an adipocyte-specific gene, in NIH-3T3 cells, rosiglitazone markedly induced its expression in NIH-3T3 cells expressing PPARγ, but not in NIH-3T3 cells infected with the control virus (Fig. 1D).
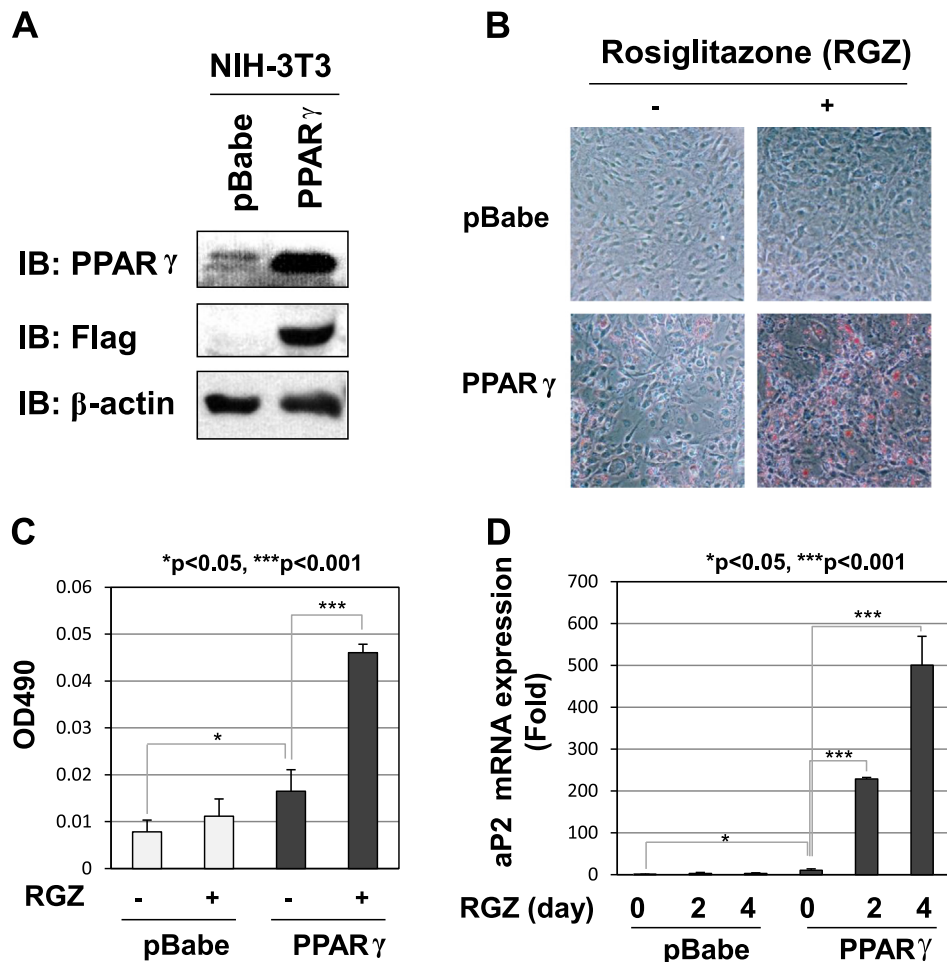
Fig. 1. Rosiglitazone induces the accumulation of lipid droplets and expression of aP2 in NIH-3T3 cells expressing PPARγ. (A) NIH-3T3 cells were infected with an empty virus (pBabe Puro) and retrovirus expressing PPARγ-Flag. Whole cell lysates were immunoblotted with an anti-PPARγ, anti-Flag, or anti-β-actin antibody. (B) Infected NIH-3T3 cells were treated with rosiglitazone (RGZ) (1 μM) for 5 days. Cells were stained with Oil-Red O. (C) The intensity of Oil-Red O staining was quantified by measuring absorbance at 490 nm (A_{490}). Values are given as the mean ± standard deviation (SD) of three independent experiments. Data were analyzed using the Student's t-test. * and *** indicate $p < 0.05$ and $p < 0.001$, respectively. (D) Infected NIH-3T3 cells were treated with RGZ (1 μM) for the indicated periods. Total RNA was prepared and the mRNA expression of aP2 was analyzed by quantitative real-time PCR. β-actin mRNA was analyzed as an internal control. Values are the mean ± S.D. of three independent experiments. * and *** indicate $p < 0.05$ and $p < 0.001$, respectively.

3.2. A proline-type fullerene derivative inhibited the accumulation of lipid droplets and expression of aP2 in NIH-3T3 cells expressing PPARγ

In order to investigate the effects of fullerene derivatives on adipocyte differentiation, we investigated whether fullerene derivatives such as the bis-malonic acid fullerene derivative (M) and three kinds of proline-type fullerene derivatives (P1, P2, and P3) affected adipogenesis-like lipid accumulation in NIH-3T3 cells expressing PPARγ (Fig. 2A).

The bis-malonic acid fullerene derivative (M) had no effect on rosiglitazone-induced lipid accumulation. Two of the proline-type fullerene derivatives, which have two carboxy groups (P1, P2), had no effect on rosiglitazone-induced lipid accumulation, whereas the proline-type fullerene derivative with three carboxy groups (P3) significantly inhibited it (Fig. 2B and C). P3 fullerene significantly inhibited the rosiglitazone-induced expression of aP2 mRNA in a dose-dependent manner. On the other hand, the other fullerenes, M, P1, and P2, failed to inhibit the expression of aP2 induced by rosiglitazone (Fig. 2D).

3.3. A proline-type fullerene derivative inhibited the MDI-induced accumulation of lipid droplets and expression of aP2 in 3T3-L1 cells

We next examined whether P3 fullerene inhibited the differentiation of 3T3-L1 preadipocytes into adipocytes. The effects of P3

fullerene on the adipogenesis of 3T3-L1 cells were assessed by staining lipid droplets with Oil-Red O, and lipid contents were quantified by measuring absorbance at 490 nm. P3 fullerene significantly inhibited MDI-induced lipid accumulation in 3T3-L1 cells (Fig. 3A and B). It also markedly inhibited MDI-induced aP2 expression (Fig. 3C), indicating that P3 fullerene prevents adipogenesis by inhibiting the activation of PPARγ.

3.4. A proline-type fullerene derivative had no effect on the expression of C/EBPδ, C/EBPβ, or PPARγ during the differentiation of 3T3-L1 cells into adipocytes

We next investigated the effects of P3 fullerene on the expression of a series of transcription factors required for adipogenesis such as C/EBPs and PPARγ during adipogenesis by performing quantitative RT-PCR and immunoblotting. The expression of C/EBPδ and C/EBPβ mRNAs was induced 1 day after the treatment with MDI, and the expression of PPARγ mRNA was induced thereafter. P3 fullerene had no effect on the MDI-induced expression of C/EBPδ mRNA, C/EBPβ mRNA, or PPARγ mRNA (Fig. 4A). Consistent with mRNA expression levels, P3 fullerene had no effect on the MDI-induced protein expression of C/EBPδ, C/EBPβ, or PPARγ (Fig. 4B). Since PPARγ mRNA is known to be induced by C/EBPδ and C/EBPβ [7–9], P3 fullerene may have failed to
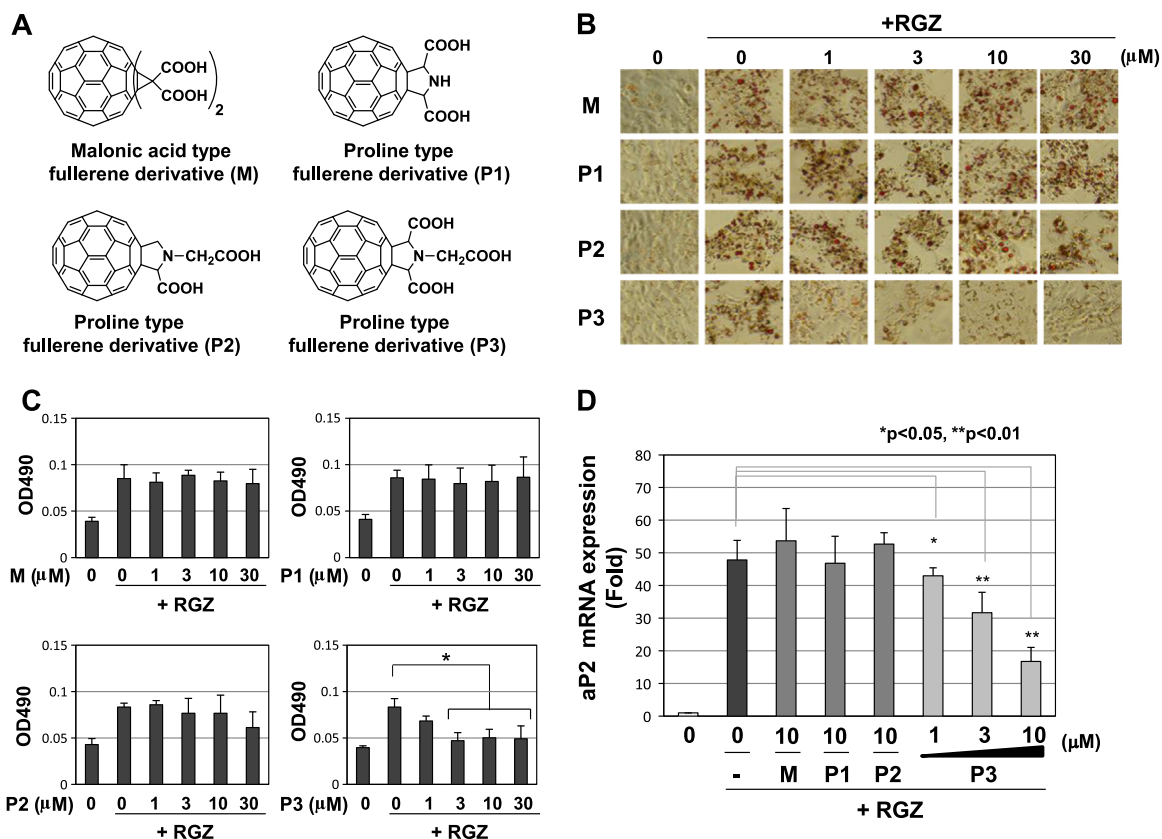
Fig. 2. A proline-type fullerene derivative inhibits the rosiglitazone-induced accumulation of lipid droplets and expression of aP2 in NIH-3T3 cells expressing PPARγ. (A) Structures of the bis-malonic acid fullerene derivative (M) and proline-type fullerene derivatives (P1, P2, and P3). (B) NIH-3T3 cells expressing PPARγ were treated with rosiglitazone (RGZ) (1 μM) in the presence of fullerene derivatives (1, 3, 10, and 30 μM) for 5 days. Cells were stained with Oil-Red O. (C) Oil-Red O staining was quantitatively evaluated by measuring A_{490}. Values are the mean ± S.D. of three independent experiments. Data were analyzed using the Student's t-test. * indicates $p < 0.05$. (D) NIH-3T3 cells expressing PPARγ were treated with RGZ (1 μM) in the presence of fullerene derivatives for 4 days. Total RNA was prepared and the mRNA expression of aP2 was analyzed by quantitative real-time PCR. β-actin mRNA was analyzed as an internal control. Values are the mean ± S.D. of three independent experiments. Data were analyzed using the Student's t-test. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.

inhibit not only the expression of C/EBPδ and C/EBPβ, but also their activities.

4. Discussion

Fullerenes may be functionally derivatized by numerous types of chemical modifications, and have been investigated as novel tools to diagnose, monitor, and treat various diseases [11–14]. A previous study showed that proline-type fullerene derivatives exhibited anti-viral activity against HIV and Hepatitis C [19]. Proline-type fullerene derivatives were recently found to inhibit the endonuclease activity of PA, a subunit of influenza A virus, and this inhibitory effect induced resistance to Influenza A viral infection [22]. These findings indicate the potential of proline-type fullerene derivatives as novel anti-viral drugs in the future. In the present study, we showed that one of the proline-type fullerene derivatives examined significantly inhibited the adipogenesis of 3T3-L1 cells (Fig. 3). This is the first study to suggest the potential of proline-type fullerene derivatives in the treatment or prevention of obesity. The proline-type fullerene derivative, P3 fullerene specifically inhibited the activation of PPARγ, which is the master transcriptional regulator of adipocyte differentiation and controls the expression of a series of genes involved in lipid metabolism (Figs. 2 and 3). A comparison of the structures of the three kinds of proline-type fullerene derivatives tested in this study revealed that three carboxy groups were necessary for fullerene derivatives to exhibit

inhibitory activity against the activation of PPARγ and accumulation of lipid droplets (Fig. 2).

Xiao et al. previously reported that a highly hydroxylated fullerene derivative inhibited H_2O_2-stimulated lipid accumulation in 3T3-L1 cells [23]. These findings also support the application of a fullerene derivative as an anti-obesity drug; however, the inhibitory mechanism underlying adipogenesis by highly hydroxylated fullerene derivatives has not yet been elucidated in detail, and appears to differ from that of proline-type fullerene derivatives. In obesity, ROS are generated from over-expanded adipose tissue [24,25]. Highly hydroxylated fullerene derivatives are highly reactive with radical species and inhibit lipid uptake-induced ROS generation in OP9 preadipocytes, a reduction that appears to correlate with decreases in the expression of PPARγ [23]. However, it currently remains unclear whether oxidative stress is involved in adipogenesis or the induction of PPARγ expression. On the other hand, the expression level of PPARγ was not changed in 3T3-L1 cells treated with P3 fullerene (Fig. 4). In addition, although a bis-malonic acid fullerene derivative exhibited potent anti-oxidant activity [17], it failed to inhibit adipogenesis or the activation of PPARγ (Fig. 2). Therefore, the inhibitory effects of P3 fullerene on adipogenesis are unrelated to its reactivity with ROS.

Adipogenesis is a unique biological event, which is processed by the sequential activation of three transcription factors, C/EBPβ, C/EBPα, and PPARγ through the induction of their expressions [5–7]. Although the endogenous ligand for PPARγ is not identified, P3 fullerene suppresses the rosiglitazone-induced lipid accumulation and mRNA expression of aP2 (Fig. 2), suggesting that P3 fullerene
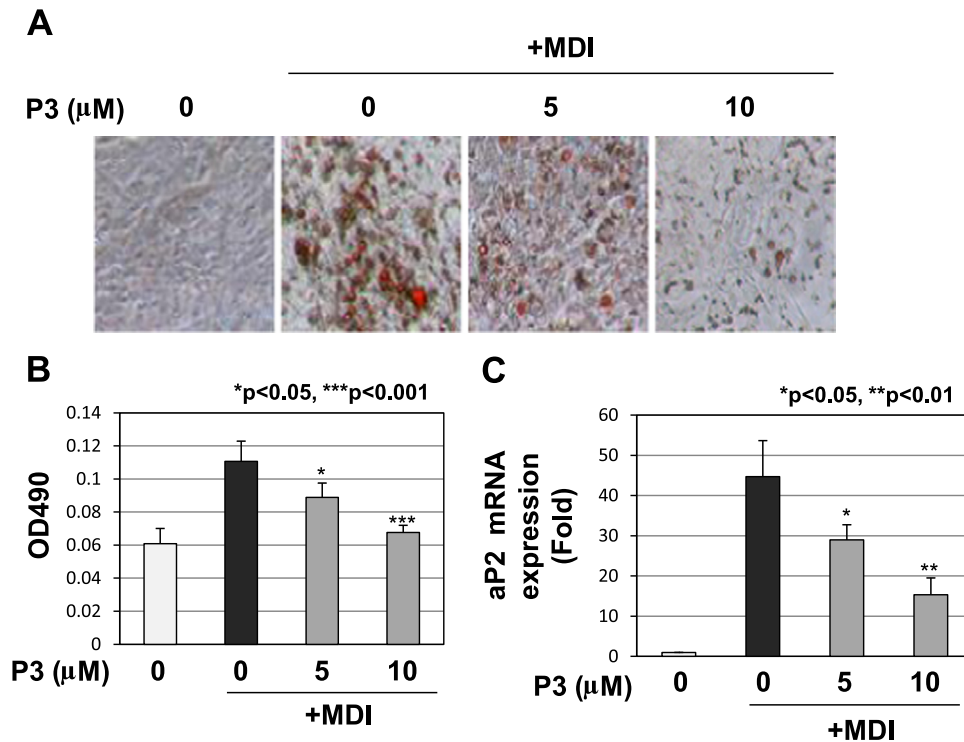
Fig. 3. A proline-type fullerene derivative inhibits the MDI-induced accumulation of lipid droplets and expression of aP2 in 3T3-L1 cells. 3T3-L1 cells were treated with a proline-type fullerene derivative (P3) (10 μM) and MDI. (A) Cells were stained by Oil-Red O on day 6 after the treatment with MDI. (B) Oil-Red O staining was quantified by measuring A_{490}. Values are the mean ± S.D. of three independent experiments. Data were analyzed using the Student's t-test. * and *** indicate $p < 0.05$ and $p < 0.001$, respectively. (C) Total RNA was prepared on day 6 after the treatment with MDI. The expression of aP2 mRNA was analyzed by quantitative real-time PCR. β-actin mRNA was analyzed as an internal control. Values are the mean ± S.D. of three independent experiments. Data were analyzed using the Student's t-test. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.
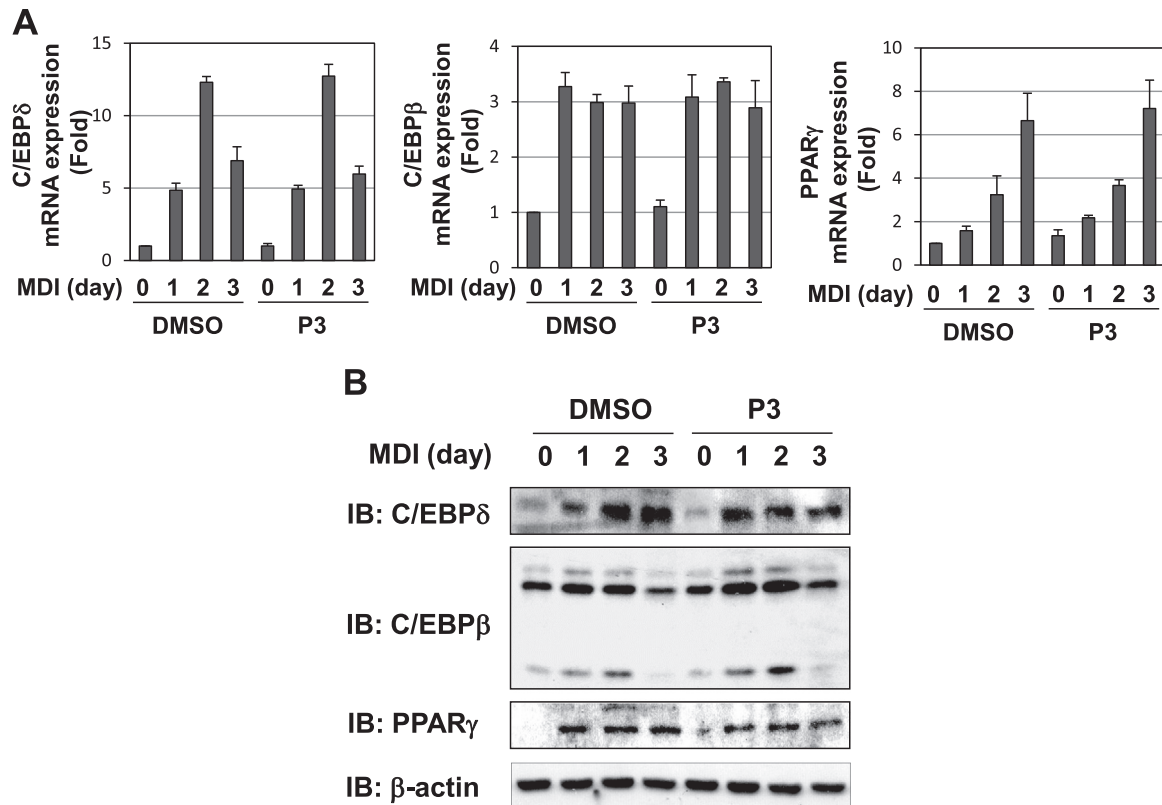

Fig. 4. A proline-type fullerene derivative has no effect on the expression of C/EBPs or PPARγ in 3T3-L1 cells. (A, B) 3T3-L1 cells were treated with a proline-type fullerene derivative (P3) (10 μM) and MDI for 3 days. (A) Total RNA was prepared and the expression of C/EBPδ, C/EBPβ, and PPARγ mRNAs was analyzed by quantitative real-time PCR. β-actin mRNA was analyzed as an internal control. Values are the mean ± S.D. of three independent experiments. (B) Whole cell lysates were immunoblotted with an anti- C/EBPδ, anti-C/EBPβ, anti-PPARγ, or anti-β-actin antibody.

seems to directly target PPARγ. The promoter region of aP2 includes the binding elements for PPARγ, therefore aP2 is understood as a direct target gene of PPARγ [8,9]. Transcription induced by PPARγ is also reported to be accelerated by histone modification, such as acetylation induced by P300/CBP and PGC-1α [26]. In the current study, we do not have enough explanation how P3 fullerene inhibits PPARγ-induced transcription. P3 fullerene could suppress the PPARγ-caused transcription mediated three possible mechanisms such as (1) P3 fullerene may block the ligand binding to PPARγ, (2) P3 fullerene may inhibit the DNA binding activity of PPARγ, and (3) P3 fullerene may suppress the transcriptional activation by PPARγ. To clarify the molecular mechanism, by which P3 fullerene inhibits PPARγ-induced transcription, the detailed analysis such as *in vitro* ligand binding assay and DNA binding assay would be suitable. In addition, the analysis of protein complexes including PPARγ would provide the molecular basis how PPARγ is activated, and these knowledge would help us to understand how P3 fullerene inhibits the adipogenesis.

In the future study, it is important to determine whether P3 fullerene exhibits anti-obesity activity *in vivo*. Although PPARγ was originally discovered as a pivotal regulator of adipocyte differentiation, it has been reported to have various functions. The expression of PPARγ has been detected in various cancer cells including breast, colon, and non-small cell lung cancers, where PPARγ regulates cell proliferation, differentiation, and apoptosis [27–31]. Previous studies reported that ligand-induced PPARγ activation inhibited the invasion and metastasis of breast cancer cells [32,33]. Furthermore, the *in vivo* administration of PPARγ ligands prevented the development of leptin-induced MCF-7 tumor xenografts in nude mice [34]. Previous studies also reported that heterozygous mice in which one allele of PPARγ was deleted (PPARγ +/−) were more susceptible to experimentally induced arthritis and allergic encephalomyelitis, suggesting that PPARγ has anti-inflammatory functions [35,36]. These findings suggest that the administration of proline-type fullerenes *in vivo* will increases the risk of exacerbating cancer and excess inflammation responses through the inhibition of PPARγ. Therefore, the development of tissue-specific PPARγ inhibitors is needed for more effective and safer therapies against obesity.

Acknowledgments

Appendix A. Transparency Document

Transparency Document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.01.001.

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