Effects Of PPARγ2 Pro12Ala Variant On Adipocyte Phenotype Dependent Of DHA

Background: Peroxisome proliferator-activated receptor γ2 (PPARγ2) plays a critical role in the regulation of adipocyte differentiation and adipocytokine production. The Pro12Ala variant is the most common mutation in the PPARγ2 gene. Its effect appears to be sensitive to dietary factors, such as docosahexaenoic acid (DHA) level. The purpose of this study was to investigate the interaction effect between PPARγ2 Pro12Ala variant and DHA on the phenotypes of adipocytes.

Methods: We generated stable 3T3-L1 cell lines expressing wild-type PPARγ2 or PPARγ2 Pro12Ala variant. These two cell lines were cultured with different concentrations of DHA (0, 50, 200 μmol/L). Then Oil red O staining was used to observe cell differentiation and the degree of lipid accumulation, TUNNEL assay was used to detect cell apoptosis, and ELISA assays were used to detect the changes of TNF-α, resistin and adiponectin levels in cell culture supernatant.

Results: PPARγ2 Pro12Ala variant reduced lipid droplet accumulation in 3T3-L1 preadipocytes treated with or without 50 μmol/L DHA, but not with 200 μmol/L DHA, compared to that of wild-type PPARγ2. PPARγ2 reduced resistin production and increased adiponectin production in 3T3-L1 adipocytes, whereas PPARγ2 Pro12Ala variant diminished these effects. However, the absence of DHA blocked PPARγ2 Ala12 variant-induced effects on adiponectin production. There was no significant difference in TNF-α secretion between wild-type PPARγ2 and PPARγ2 Pro12Ala cells whether with or without DHA.

Conclusion: These results indicated that the effects of PPARγ2 Pro12Ala variant were dependent on DHA concentration.

Keywords: peroxisome proliferator-activated receptor γ2, Pro12Ala variant, docosahexaenoic acid, adipocyte, differentiation, adiponectin

Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ), particularly the PPARγ2 isoform, is a ligand-dependent nuclear receptor highly expressed in adipose tissue. Activation of PPARγ2 regulates adipocyte differentiation, triglyceride storage, and some metabolic effects, including glucose homeostasis and insulin sensitivity.1,2

The Pro12Ala variant of the PPARγ2 gene is common (3–14%)3 and can cause a moderate decrease in its transcriptional activity and adipogenic potential.4 Human population studies on PPARγ2 gene Pro12Ala polymorphism showed the association between this variant and reduced weight gain and reduced risk of type 2 diabetes.5,6 Moreover, the Pro12Ala variant appears to be sensitive to environmental effects, such as dietary factors.7–9

Long-chain n-3 polyunsaturated fatty acids, such as docosahexaenoic acid (DHA), have been reported to reduce adiposity by preventing fat accretion and
improve glycolipid metabolic disorders in high-fat diet-fed rats.\textsuperscript{10} Additionally, a randomized clinical trial showed that DHA exhibited anti-inflammatory effects.\textsuperscript{11,12}

Thus, in this study, we aimed to examine the gene-environment interactions between PPAR\textsubscript{2} Pro12Ala variant and DHA in 3T3-L1 preadipocytes. We generated stable 3T3-L1 cell lines expressing wild-type PPAR\textsubscript{2} or PPAR\textsubscript{2} Pro12Ala variant. Then, the effects of wild-type PPAR\textsubscript{2} and PPAR\textsubscript{2} Pro12Ala variant on the differentiation, apoptosis, and adipocytokine levels were investigated in 3T3-L1 preadipocytes treated with or without different concentrations of DHA.

Materials And Methods

Cell Culture
HEK293T cells were obtained from the American type culture collection (ATCC). 3T3-L1 preadipocytes were obtained from the Chinese Academy of Sciences Cell Bank. All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10\% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 mg/mL) in a humidified incubator with 5\% CO\textsubscript{2} (v/v) at 37 °C.

Construction Of Lentivirus Plasmids Containing PPAR\textsubscript{2} Pro Or PPAR\textsubscript{2} Ala Gene
Wild-type PPAR\textsubscript{2} and PPAR\textsubscript{2} Pro12Ala genes were polymerase chain reaction (PCR)-amplified from human adipose tissue cDNA library. The PCR products were subcloned into the pMD18-T vectors (Takara), and the Pro12Ala variant was confirmed by sequencing (Shanghai Boshang Biotechnology Co. Ltd.). Then, the products were cloned into pLVTHM via restriction digestion (EcoRI/XhoI) and T4 DNA ligase (TOYOBO) to construct the pLVTHM-PPAR\textsubscript{2} Pro and pLVTHM-PPAR\textsubscript{2} Ala plasmids.

Preparation Of Lentivirus
Lentiviral supernatants were produced in HEK293T cells by co-transfection of 20 μg of transfer vectors harboring the indicated genes (empty vector, pLVTHM-PPAR\textsubscript{2} Pro, or pLVTHM-PPAR\textsubscript{2} Ala), 10 μg of pRsv-REV, 15 μg of pMDlg-pRRE, and 7.5 μg of pMD2G, according to the manufacturer’s protocol. Supernatants containing lentivirus were collected at 48 h after transfection. Cell debris was removed by centrifugation at 4000 \times \text{g} for 10 min at 4 °C, followed by filtration through a 0.45 μm polyethersulfone membrane filter unit. Then, the virus was concentrated at 72,000 \times \text{g} for 120 min at 4 °C.

Generation Of Stable Cell Lines

3T3-L1 cells were seeded on a 24-well plate at a density of 5 × 10\textsuperscript{4} cells/well. Then, in the presence of polybrene (1 μg/mL), cells were infected with pLVTHM-GFP lentivirus (LV-vector group), pLVTHM-PPAR\textsubscript{2} Pro lentivirus (LV-PPARG2 group), or pLVTHM-PPAR\textsubscript{2} Ala lentivirus (LV-PPARG2 P12A group). After 24 h incubation at 37 °C, the culture medium was replaced with a fresh complete medium without polybrene. At 72 h post-infection, stably transfected cell lines were selected using puromycin (Sigma-Aldrich; Merck Millipore) at a concentration of 1 μg/mL for 7 days.

Quantitative RT-PCR Assay

Total RNA was extracted using Trizol (Invitrogen), according to the manufacturer’s instructions. cDNA was synthesized from 1 μg of RNA using a cDNA reverse transcription kit with RNase inhibitor (MBI Fermentas). Real-time PCR was performed in a thermal cycler (ABI) using Quant qRT-PCR (SYBR Green I) kit (TIANGEN). The \(\Delta\)CT method was used to measure the relative expression. Results were normalized to that of a reference gene (β-actin). The primer sequences were as follows: PPAR\textsubscript{2} (5′-GGAGCCCAAGTTTGAGTTTGCTGT-3′, 5′-AGGGCTTGTAGCCGTTCTTGAG-3′) and β-actin (5′-TGTGATGGTAGAGTTGTCAGAA-3′, 5′-TGTGGTCAGATCTTTCTCCATGT-3′).

Fatty Acid Treatments

On the first day of cell differentiation, the same number of 3T3-L1 cells were inoculated into 24-well plates. 3T3-L1 cells were treated with different concentrations of DHA (0, 50, or 200 μmol/L) and MDI (Sigma) containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/mL insulin.

Oil Red O Staining

After incubation with DHA for 6 h, the cells were washed three times with phosphate-buffered saline (PBS), fixed with 10\% formaldehyde for 30 min, and then stained with 0.5\% Oil Red O (Sigma) for 20 min at 20 °C. Red-stained adipocytes were observed under a phase-contrast microscope (Olympus). To quantify Oil Red O levels, 2.5 mL of 100\% isopropanol was added to each well. After shaking at room temperature for 5 min, the optical density (OD) of samples was measured at 500 nm using a spectrophotometer (UV-765).
TUNEL Assay
A one-step TUNEL cell apoptosis detection kit (Ggreen fluorescence) was purchased from Beyotime Biotechnology Research Institute. Briefly, cells cultured with DHA for 2 h were fixed with 4% paraformaldehyde for 30–60 min at room temperature. After washing with PBS, the cells were permeabilized with PBS containing 0.1% Triton X-100 on ice for 2 min. Then, they were stained with a freshly-prepared TUNEL detection solution (20 μL of TdT enzyme, 480 μL of the fluorescent labeling solution, and 500 μL of the TUNEL detection solution) in dark at 37 °C for 60 min, followed by sealing using the antifluorescence quenching sealing fluid. Finally, apoptosis rates were evaluated using a fluorescence microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)
Adiponectin ELISA kit was purchased from B-Bridge International Corporation. Tumor necrosis factor-α (TNF-α) and resistin ELISA kits were obtained from Quantikine International Corporation. Tumor necrosis factor-α (TNF-α) and resistin ELISA kits were obtained from Quantikine International Corporation. Cytokine levels were measured by taking cell medium, according to the manufacturer’s instructions.

Statistical Analysis
Values are expressed as the means ± standard error of the mean (SEM). Data were analyzed using SPSS package program version 22.0. One-way analysis of variance followed by Student-Newman-Keuls (SNK) post-hoc test was used to compare the responses among different groups. A P-value < 0.05 was considered statistically significant.

Results
Generation Of Stable Cell Lines
To investigate the effects of PPARγ2 genotypes in 3T3-L1 cells, we generated stable cell lines expressing wild-type PPARγ2 (Lv-PPARG2) or PPARγ2 Pro12Ala variant (Lv-PPARG2 P12A). Plasmids containing the Pro12Ala variant were confirmed by sequencing. The presence of the CCA→GCA mutation (Pro/Ala) at codon 12 of PPARγ2 exon B without additional changes in the coding sequence was verified. The expression of PPARγ2 in stable cell lines was verified by real-time PCR assay. The expression of PPARγ2 in 3T3-L1 cells transfected with Lv-PPARG2 or Lv-PPARG2 P12A increased by 10 fold, compared to that in 3T3-L1 cells transfected with Lv-vector.

Effects Of PPARγ2 Pro12Ala Variant On Lipid Droplet Formation In DHA-Treated 3T3-L1 Adipocytes
The effects of PPARγ2 genotypes on 3T3-L1 preadipocyte differentiation were investigated. Lv-vector, Lv-PPARG2, or Lv-PPARG2 P12A-transfected 3T3-L1 cells were treated with different concentrations of DHA. Cells were stained with Oil Red O to evaluate the degree of lipid accumulation. As shown in Figure 1, overexpression of PPARγ2 increased lipid accumulation in 3T3-L1 cells treated with 0 or 50 μmol/L DHA, whereas overexpression of PPARγ2 Pro12Ala variant reduced lipid accumulation, compared to that of PPARγ2. However, at a concentration of 200 μmol/L DHA, there was no statistical difference among the control, PPARγ2 Pro12, and PPARγ2 Pro12Ala-expressing adipocytes.

Effects Of PPARγ2 Pro12Ala Variant On Adipocyte Apoptosis In DHA-Treated 3T3-L1 Adipocytes
We then evaluated apoptosis ratios in different groups. As shown in Figure 2, overexpression of wild-type PPARγ2 or PPARγ2 Pro12Ala variant did not affect apoptosis in 3T3-L1 adipocytes treated with 0 or 50 μmol/L DHA. However, in both wild-type PPARγ2 and PPARγ2 Pro12Ala variant-expressing adipocytes treated with 200 μmol/L DHA, apoptosis ratios were reduced, compared to that in the control group. At all DHA concentrations, there was no statistical difference in apoptosis ratios between 3T3-L1 adipocytes overexpressing wild-type PPARγ2 and PPARγ2 Pro12Ala variant.

Effects Of PPARγ2 Pro12Ala Variant On Inflammatory Cytokine Levels In DHA-Treated 3T3-L1 Adipocytes
Furthermore, we examined the effects of PPARγ2 genotypes on the secretion of inflammatory cytokines in 3T3-L1 adipocytes. As shown in Figure 3, overexpression of wild-type PPARγ2 decreased the secretion of proinflammatory cytokines and resistin from 3T3-L1 adipocytes treated with or without DHA. Moreover, overexpression of PPARγ2 Pro12Ala variant partially abolished these effects (Figure 3B). On the contrary, overexpression of wild-type PPARγ2 increased the secretion of adiponectin from 3T3-L1 adipocytes treated with or without DHA. Similarly, overexpression of PPARγ2 Pro12Ala variant in DHA-treated cells attenuated these effects. However, the absence of DHA blocked PPARγ2 Ala12 variant-induced
effects on adiponectin production (Figure 3C). However, there was no significant difference in TNF-α secretion between wild-type PPARγ2 and PPARγ2 Pro12Ala-expressing adipocytes, regardless of DHA treatment (Figure 3A).
Dissussion

PPARγ2 is a nuclear receptor involved in lipid metabolism, adipocyte differentiation, and proliferation. A missense mutation (CCGPro-GCGAla) at codon 12 is a common variant of PPARγ2 gene. Meta-analyses showed that PPARγ2 Pro12Ala variant correlated with improved insulin...
Another clinical study verified that PPAR-γ2 Pro12Ala variant had modest protective effects against the development of type 2 diabetes.\(^\text{14,15}\)

In our study, overexpression of wild-type PPARγ2 gene resulted in increased lipid droplet accumulation and adipocyte differentiation. PPARγ2 Pro12Ala variant with or without 50 μmol/L DHA treatment attenuated these effects, suggesting that PPARγ2 Pro12Ala variant might correlate with lower risk of obesity. However, there was no significant difference in lipid droplet accumulation between wild-type PPARγ2 and PPARγ2 Pro12Ala1-expressing adipocytes treated with 200 μmol/L DHA, indicating that the effects of PPARγ2 Pro12Ala variant was dependent on the concentration of DHA to some extent. Meantime, our results showed that DHA can induce apoptosis of adipocytes and this effect becomes more obvious with the increase of DHA concentration. This may also be the reason why there is no statistically significant difference in 200μmol/L DHA group.

However, overexpression of PPARγ2 did not affect apoptosis of adipocytes except when incubated with high concentration (200μmol/L) of DHA. And PPARγ2 Pro12Ala variant showed no effect on apoptosis of adipocytes.

It has been reported that some adipocytokines, such as resistin, TNF-α, and adiponectin, are under the transcriptional control of PPARγ2. Resistin belongs to the family of cysteine-rich proteins, called resistin-like molecules.\(^\text{17}\) The expression of resistin positively correlated with body fat, insulin, glucose, and triglyceride levels in high-fat diet-fed mice.\(^\text{18}\) Additionally, a PPARγ agonist was shown to improve insulin sensitivity via downregulation of resistin expression.\(^\text{19}\) PPARγ also regulates the circulating levels of adiponectin, where the effects of PPARγ on adiponectin production are partially attributed to the direct activation of adiponectin gene transcription.\(^\text{20}\) Adiponectin expression and its signaling pathway are directly regulated by PPARγ.

In line with the results of previous studies, we showed that overexpression of PPARγ2 gene reduced TNF-α and resistin production and increased adiponectin production. However, overexpression of PPARγ2 Pro12Ala variant attenuated these effects. It is noteworthy that a significant difference in adiponectin level between wild-type PPARγ2 and PPARγ2 Pro12Ala1-expressing cells was only observed in cells treated with DHA (50 and 200 μmol/L). There was no significant difference in adiponectin level between wild-type PPARγ2 and PPARγ2 Pro12Ala1-expressing DHA-untreated cells. Additionally, PPARγ2 Pro12Ala variant showed no effect on TNF-α secretion in adipocytes, compared to that of wild-type PPARγ2.

In summary, our study showed that overexpression of PPARγ2 gene in preadipocytes resulted in increased lipid droplet accumulation and adipocyte differentiation, and exhibited anti-inflammatory effects by reducing proinflammatory cytokine (TNF-α and resistin) secretion and increasing anti-inflammatory cytokine (adiponectin) production. Most of these effects, except for TNF-α secretion, were attenuated owing to the overexpression of PPARγ2 Pro12Ala variant. Moreover, the effects of PPARγ2 Pro12Ala variant varied at different concentrations of DHA treatment. The effects of PPARγ2 Pro12Ala variant on adiponectin secretion were dependent on the presence or absence of DHA treatment. Additionally, the effect of PPARγ2 Pro12Ala variant were dependent on gene-environment interactions. Several clinical studies also found that the regulation of body fat by PPARγ2 Pro12Ala variant was affected by dietary conditions.\(^\text{21,22}\) However, further studies are needed to clarify the exact effects of PPARγ2 Pro12Ala variant under different dietary conditions.

**Abbreviations**

ATCC, American type culture collection; DHA, docosahexaenoic acid; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor; SNK, Student-Newman-Keuls; TNF-α, tumor necrosis factor-α.

**Author Contributions**

JL and LYZ designed the study. RHW, ZPD and SX performed the experiments. RHW analyzed the data and wrote the manuscript. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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**Disclosure**

The authors report no conflicts of interest in this work.
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