Orphan Receptor GPR50 Improves Inflammation and Insulin Signaling in 3T3-L1 Preadipocyte

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Abstract

Purpose: The goal of this study was to investigate the effect of orphan G Protein-Coupled Receptor 50 (GPR50) receptor on inflammation and insulin signaling in 3T3-L1 preadipocyte.

Subjects and Methods: A high-fat diet (HFD)-induced obesity-T2DM (Type 2 Diabetes Mellitus) mouse model was used in this research, and high expression of GPR50 in mouse adipose tissue was screened by microarray technology. Expression of GPR50 in 3T3-L1 cell line and obesity-T2DM mouse adipose tissue was confirmed. To gain more insight into the potential role of this new target in obesity-associated IR development, a GPR50 knockout cell line was constructed in 3T3-L1 cell line. Inflammatory cytokine levels and insulin signaling pathways in the GPR50 knockout 3T3-L1 cell line were determined by quantitative real-time polymerase chain reaction analysis and western blot.

Results: GPR50 expression was significantly increased in adipose tissue of obesity-T2DM mice. GPR50 deficiency increased inflammation in 3T3-L1 cells. In addition, GPR50 deficiency induced the phosphorylation of AKT and insulin receptor substrate (IRS) 1. Furthermore, GPR50 knockout 3T3-L1 cell line had suppressed PPAR- γ expression.

Conclusions: These data demonstrated a novel target GPR50 can affect inflammation and insulin signaling in adipocytes. Furthermore, the effects are mediated through the regulation of insulin signaling and PPAR- γ expression.

Introduction

Type 2 diabetes (T2DM) is a metabolic disease which is widely prevalent in the world. It is characterized by insulin secretion deficiencies and systemic insulin resistance (IR) in adipose tissue, skeletal muscle, and liver. (Rachdaoui 2020) Although the mechanism of T2DM is not yet fully known, inflammation and insulin resistance play a central role in the pathogenesis of T2DM.

According to recent analysis, the role of adipose tissue is crucial in the progression of diabetes. T2DM increases the mass of visceral adipose tissue (Murai et al. 2018) and secretes various inflammatory cytokines such as interleukin (IL)-1 β , monocyte chemoattractant protein 1 (MCP-1), and IL-6 that triggers chronic, low-grade inflammation. (Lackey and Olefsky 2016) In a proinflammatory environment, insulin signaling pathways are activated, leading to a decreased insulin sensitivity in cells. Ample evidence suggests that inflammatory mediators directly inhibit insulin signaling, with c-Jun N-terminal kinase (JNK), inhibitor of nuclear factor kappa-B kinase beta (IKK β) and protein kinase C γ (PKC γ) blocking insulin signaling by phosphorylating insulin receptors and insulin receptor substrate (IRS) proteins at inhibitory sites. (Guo 2014; Matulewicz and Karczewska-Kupczewska 2016) Nuclear factor kappa-B (NF- κ B) can inhibit the expression of insulin signaling pathway components, including, IRS1 and protein kinase B (AKT/PKB). (Zatterale et al. 2019; Yaribeygi et al. 2019)

Multiple metabolic pathways are responsible for insulin resistance in obesity and type 2 diabetes mellitus (T2DM). (Haeusler, McGraw, and Accili 2018) Insulin signaling is thought to be regulated by the insulin receptor and its substrates, phosphatidylinositol 3-kinase (PI3K), and AKT/protein kinase B (PKB). (Yang, Vijayakumar, and Kahn 2018; Manning and Toker 2017) Insulin signaling is composed of two signaling pathways related to insulin receptor. The phosphatidylinositol 3-kinase (PI3K)-AKT pathway and the Ras-mitogen activated protein kinase (MAPK) pathway, which also interacts with the PI3K-AKT pathway. (Yang, Vijayakumar, and Kahn 2018; Manning and Toker 2017) They can both be activated by the insulin/insulin receptor substrate-1 (IRS-1) system in insulin resistance. According to the available findings, phosphorylation of IRS1 is strongly correlated with insulin resistance. (Copps and White 2012)

Adipose tissue is an important initial site of IR, and peroxisome proliferators-activated receptor- γ (PPAR- γ) has a major influence on the differentiation of adipose tissue, macrophage polarization, and the secretion of adipogenic factors. (Moseti, Regassa, and Kim 2016) Moreover, PPAR- γ is an important regulator of lipid metabolism and insulin sensitivity. (Moseti, Regassa, and Kim 2016) Activated PPAR- γ heterodimerizes with retinoid X receptor- α (RXR- α) to maintain glucose

homeostasis through direct regulation of IRS-1 and glucose transporter type 4 (GLUT4).(Ahmadian et al. 2013) Loss of PPAR- γ in skeletal muscle, severe IR was shown.(Hevener et al. 2003)

G protein-coupled receptors (GPCRs) are involved in endocrine and metabolic processes as well as many other physiological processes. Many medicinal drugs, including glycolipid metabolism drugs, target GPCRs.(Rask-Andersen, Almen, and Schioth 2011) GPR50 is an orphan GPCR, which shares highest sequence homology with melatonin receptors.(Emet et al. 2016) GPR50 has shown to be an important regulator of energy metabolism in GPR50 knockout mouse.(Ivanova et al. 2008) A sequence variant study suggests that GPR50 is related to mental disorders(Watkins and Orlandi 2020) and altered lipid metabolism.(Bhattacharyya et al. 2006)

To investigate gene differential expression in mouse epididymal adipose tissue, we developed a HFD-induced obesity-T2DM mouse model. We observed a drastic increase in the expression of the orphan GPCR, GPR50, in the adipose tissue of HFD mice via gene array technology. We investigated the roles of GPR50 deficiency in obesity induced inflammation and insulin signaling. The association of GPR50 with inflammation and insulin signaling pathway was investigated by using GPR50 knockout 3T3-L1 cell line. The phosphorylation of IRS-1 and AKT were observed activated significantly in GPR50 knockout cell line. Also, we have shown that GPR50 regulates insulin signaling and PPAR- γ expression.

Materials And Methods

Cells, reagents, and treatments

The American Type Tissue Culture Collection provided the mouse preadipocyte 3T3-L1 cell line (Manassas, VA). Cells were maintained in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) and 10% bovine calf serum (GIBCO, Carlsbad, CA) at 37 °C in a 5% CO₂ incubator. In treatment experiments, 3T3-L1 cells were incubated in treatment experiments with 16.7 mM glucose or 0.25 mM palmitate for 48 h to mimic obese adipocytes.(Manna, Achari, and Jain 2017; Li et al. 2016) Antibody against IRS-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-IRS-1[Ser612], phospho-Akt[Ser473], Akt, β -actin, GAPDH and PPAR- γ were purchased from Cell Signaling Technology (Danvers, MA). GPR50 antibody was purchased from Proteintech Group, Inc (Rosemont, IL). For insulin stimulation, GPR50 knockout 3T3-L1 cells or control cells were seeded in 6-well plates. Two days after reaching confluence, cells were treated with serum starvation in DMEM for 4 h, then washed three times with PBS. Cells were incubated in DMEM containing 100 nM insulin or DMEM alone for 30 min. Finally, cells were washed 3 times and proteins were extracted for Western blot detection.

Differentiation of 3T3-L1 adipocytes

Mouse 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) with 10% fetal bovine serum (FBS, GIBCO). Two days after reaching confluence, the cells medium was modified into differentiation medium (StemPro™ Adipogenesis Differentiation Kit). The induction medium was replaced every three days until the 3T3-L1 had differentiated into mature adipocytes.

Mouse model

Eight weeks old C57BL/6J male mice were obtained from Guangdong Medical Laboratory Animal Center (Guangdong, China). The mice were randomly divided into two groups after one-week adaptation: the HFD group (n=3 from 5) and the chow group (n=3 from 5). Mice were fed high-fat diet (45% fat, Research diet D12451, New Jersey) or chow diet ad libitum for 15 weeks before the symptoms of the mice were characteristic for T2DM. The body weight was assessed every two weeks. Animal care was in compliance with the Guide for the Care and Use of Laboratory Animals of Guangdong Province. All the procedures were under the supervision and approved by the Ethics Committee for Animal Research, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences (Approval Number: SIAT-IRB-170401-YGS-RPG-A0312-01).

RNA Extraction

The epididymal fat tissues have been isolated from the mice. RNA samples were collected from the adipose tissue using Trizol reagent (Invitrogen Corporation, California) and were validated with Agilent Array platform for microarray assay and real-time PCR analysis.

Microarray Analysis

The study of microarray was performed by GMINIX (Shanghai, China) with Mouse Transcriptome Array 1.0 (Affymetrix). This microarray targets about 114,000 protein-coding transcripts. We purified RNA from three HFD and three chow mice in epididymal adipose tissues, and used a random priming process to transcribe the RNA into complementary DNA (cDNA). In the GeneChip® Hybridization Oven 645, cDNA was then fragmented, biotinylated and 5.5 µg of cDNA was hybridized to the GeneChip Mouse Transcriptome Array 1.0. The arrays were screened with the Gene Array Scanner 3000 7G (Affymetrix, California) after hybridization and washing. All the data were analyzed using the Robust Multichip Analysis (RMA) algorithm using Affymetrix default analysis settings and global scaling as a normalization process.

Quantitative real-time PCR

The total RNA was extracted by the Trizol reagent from cells and mouse adipose tissue (Invitrogen Corporation, CA). Reverse transcription and RT-PCR were performed using RT-PCR Kit (Takara, Japan). For Q-PCR, a light Cyclor (Roche, Switzerland) and SYBR Quantitative real-time PCR (Takara, Japan) kit were used. The endogenous control was used as GAPDH. Table 1 presents PCR primers used for mRNA quantitation and were synthesized from Sangon Biotech (Shanghai, China).

Enzyme-linked immunosorbent assay

GPR50 knockout 3T3-L1 or control cells were incubated with 16.7 mM glucose or 0.25 mM palmitate for 48 h as described above. Supernatants were collected centrifuge at 3 000 rpm for 5 min. The concentrations of IL-6 (Dakewe, CN), MCP-1 (Sino biological, CN) and IL-1β (R&D Systems, Minneapolis) assayed with ELISA system kit according to manufacturers' instructions. Quantitative data are presented as average concentrations in pg/ml .

Construction of GPR50 knockout cell line

To establish stable GPR50-knockdout 3T3-L1 cell line, using an online CRISPR design tool (<http://crispr.mit.edu>), GPR50 small guide RNAs (sgRNAs) is designed. The sgRNA sequence is as following:
GTTTCAGAGCTATGCTGGAAACAGCATAGCAAGTTGAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT.
For further analysis, the sequences were synthesized and annealed to form a gRNA duplex. BsmB I digested the lentiviral vector (Obio, Shanghai, China), resulting in sticky ends. The phosphorylated and annealed sgRNA was ligated to the lentiviral vector. Sequencing confirmed the right clone and the high purity plasmid was extracted. Shanghai OBiO Technology Co, Ltd completed the lentiviral packaging (Shanghai, China). The 3T3-L1 cell suspension was added to each well of a six-well plate with a density of 5×10^4 /mL, and the lentivirus was added to each well at the proper density. For monoclonal cell screening, 2 µg/mL puromycin was introduced after 72 hours of infection. 48 hours later, the screened and selected cells were divided and seeded into 96-well plates with 1 cell/well using flow cytometry. After about 2 weeks, single-cell colonies were obtained; a single cell pellet was picked up with microscope and inoculated into a new 24-well plate to begin the culture. Empty lentivector was used as a negative control. Cells infected with knockout lentivector (KO) were called KO cells, and the cells with empty lentivector infection were called Control cells. After selection, the efficiency of infection was verified by western blot.

Western blot analysis

3T3-L1 cells were lysed with a cell lysis buffer (Cell Signaling Technology, Danvers, MA) for western blot analysis and extracted protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL). Protein (30µg) was subjected to 4–10% SDS-PAGE and transferred to a membrane of PVDF (Amersham Biosciences, UK). The membranes were then blocked for 2-3 hours at room temperature with 5% (w/v) BSA. The membranes were incubated overnight at 4°C with the indicated antibodies, and then for 1 hour with horseradish peroxidase conjugated secondary antibodies., followed by

visualization with a chemiluminescence system (ECL, Amersham Biosciences, Buckinghamshire) on ChemiDoc MP System (Bio-Rad, USA), washings performed between incubations.

Statistical analysis

Quantitative real-time PCR results are presented as relative quantity to the control group, using β -actin as internal reference gene. Band densities were corrected for background and then normalized to the control (IRS1 for p-IRS1/AKT for p-AKT/ β -actin or GAPDH for PPAR- γ) signal of the same lane in western blot experiment. ELISA experiment results are normalized as relative quantity to the control group. All data were viewed as means SEM, and the differences between the two groups were measured using a two-way ANOVA, with a $p < 0.05$ presented statistically significant. All the statistical analyses were conducted using GraphPad Prism7 (GraphPad Software, California). At least three biological replicates of each experiment were performed and analyzed.

Results

GPR50 as a novel candidate target in obesity-T2DM animal adipose tissue

To systemically screen the new targets from the adipose tissue in obesity-T2DM mice, we compare two groups of male C57BL/6J mice fed standard chow diet or HFD ($n=3$ from 5 per group) for transcriptome analyses. The microarray information is available in the Omnibus Gene Expression (GSE100028). The results of our previously published paper showed that HFD mice exhibited obesity and T2DM symptoms. (Yao et al. 2019) As shown in Figure 1, we pick out 20 most significant differentially expressed genes between the chow and HFD groups. Among these genes, we have found the expression of an orphan GPCR named GPR50 was significantly increased in the adipose tissue of HFD group compare to the Chow group. Since the importance of GPCRs for discovering new targets for T2DM treatment and our experiment with GPCR study, we investigated the effect of GPR50 on T2DM.

Expression profiles of GPR50 in adipose tissue and differentiated 3T3-L1 cell line

In epididymal adipose tissue, we confirmed the GPR50 expression increasing in the HFD mice at both mRNA and protein levels compare to the Chow mice, which was consistent with the microarray results (Figure 2A, 2B, 2C). Then, we studied GPR50 expression in 3T3-L1 pre-adipocytes during the lipogenic differentiation. The results revealed that after two days of adipogenic differentiation the expression of GPR50 increased in a time-dependent manner and reached peak at 8 days (Figure 2D, 2E, 2F). These findings showed that GPR50 may be involved in adipogenic differentiation.

Construction of GPR50 knockout 3T3-L1 cell line

To further investigate the function of GPR50 in T2DM, we constructed GPR50 knock-out cell line through CRISPR/Cas9 gene editing system. The full-length sequence of GPR50 was obtained from GeneBank database. According to the general principles of gRNA design, single-guide RNAs (sgRNAs) targeting GPR50 genes were built by an online software for CRISPR design (<http://crispr.mit.edu>). Methods and procedures have shown the SgRNA sequence. The transfer vector was successfully constructed by combining sgRNA after annealing connection. To improve transfection efficiency, the transfer vector was packaged in lentiviruses and successfully transfected into 3T3-L1 cells. The stable cell lines of GPR50 gene knockout were screened by puromycin and proved by western blot as shown in Figure 3.

Inflammation increased in GPR50 deficiency 3T3-L1 cell line

Obesity is related to the pathogenesis of IR in adipose tissue due to chronic low-grade inflammation. Selected markers of adipose inflammation in GPR50 deficiency 3T3-L1 cell line were measured. We used high glucose and palmitic acid (PA) to simulate high-fat diet stimulation cellularly. As shown in Figure 4A-D, at mRNA levels, the expression of proinflammatory cytokines IL-6, MCP-1 and IL-1 β which associated with IR, were significantly higher in GPR50 $^{-/-}$ cells compared with control cells which transfected with control plasmid. Moreover, we analyzed the protein secretion of IL-6, MCP-1 and IL-1 β in the cell

supernatant by ELISA. The results were not fully consistent with the Q-PCR. IL-6 protein secretion increased with palmitic acid (PA) stimulate; MCP1 was significantly higher in GPR50^{-/-} cells used high glucose and palmitic acid (PA) to simulate. This suggests that MCP-1 maybe more important. And all these findings indicated that GPR50 could improve adipose tissue inflammation induced by high glucose and PA.

Activation of insulin signaling in GPR50 deficient 3T3-L1 cell

IR is a pathological condition frequently linked to T2DM. GPCRs are participating in the development of IR which can lead to T2DM induced by obesity.(Ridley et al. 2018) In order to evaluate the effect of GPR50 in insulin signaling pathway, protein levels of p-IRS-1/IRS-1 p-AKT/AKT were analyzed in GPR50 deficiency 3T3-L1 cells pretreated with glucose or PA by western blot. The protein expressions of p-IRS-1 was significantly enhanced in GPR50 deficiency 3T3-L1 cell line (Figure 5A-B). Although, high glucose and PA already induced phosphorylation of AKT, GPR50 deficient 3T3-L1 cell line still had significantly higher p-AKT than control group (Figure 5A-C). High glucose and PA treatment led to an interruption of insulin pathway by reducing p-IRS-1, and this effect was reversed when GPR50 was removed (Figure 5A, B). However, not like the case for p-IRS-1/IRS-1, glucose and PA increased p-AKT/AKT level significantly in 3T3-L1 cell, and even high in GPR50 knockout 3T3-L1 cell under the treatments. These findings indicate that GPR50 might be able to improve high glucose and PA activated insulin signaling through IRS-1 pathway; but, might through other pathway related or not related to GPR50 to increase AKT phosphorylation dramatically which we didn't investigate yet (Figure 5A, C). To further evaluate the effect of GPR50 on insulin pathway, we challenged the insulin signaling assay with insulin stimulation. As shown in Figure 7, insulin stimulation will increase phosphorylation levels on the AKT and insulin receptor substrate (IRS)1. But the protein expressions of p-IRS-1 and p-AKT was significantly decreased in GPR50 knockout 3T3-L1 cell line (Figure 7). Not like the PA and high glucose stimulation, p-IRS-1/IRS-1 and p-AKT/AKT level significantly induced in 3T3-L1 cell, and significantly inhibited in GPR50 knockout 3T3-L1 cell. These findings also indicate that GPR50 might be able to inhibit insulin activated insulin signaling through IRS-1/AKT pathway, although PA and insulin stimulate the insulin pathway in opposite ways.

GPR50 enhanced transcription factor PPAR- γ expression

PPAR- γ is an essential transcription factor expressed mainly in mammalian adipose tissue and other tissues for cell differentiation. PPAR- γ involves in modulating of insulin sensitivity. As shown in Figure 6, deficiency of GPR50 inhibited PPAR- γ expression at mRNA and protein level and also related to activated insulin signaling. However, treatment with high level glucose or PA had no effect on the expression of mRNA or protein levels of PPAR- γ in control 3T3-L1 cell. More interestingly, with insulin stimulation, we also found that PPAR- γ expression was inhibited in GPR50 knockout cell lines (Figure 7). These results showed that GPR50 could still regulate the expression of PPAR- γ under insulin stimulation, and the regulation was consistent with PA and high glucose stimulation, which were conducive to improving insulin signaling.

Discussion

GPR50 was screened in HFD-induced mice adipose tissue by gene microarray technology, suggesting that it may be functional in the metabolism and development of T2DM. Previous studies have shown that GPR50 is closely related to energy expenditure and feeding behavior.(Ivanova et al. 2008) GPR50 knockout mice have higher metabolic rates, less fat accumulation, and are partly resistance to diet-induced obesity. These data suggest that GPR50 is a regulator of energy metabolism.(Ivanova et al. 2008)

T2DM and IR are connected by low-grade inflammation in adipose tissue.(Lackey and Olefsky 2016) As shown in Figure 4, we used high glucose and palmitic acid (PA) to simulate high-fat diet stimulation cellularly. We found that GPR50 could inhibit IL-6, IL-1 β and MCP-1 in mRNA level, but not in protein level. The IL-6 protein secretion increased with palmitic acid (PA) stimulate, the protein concentration of IL-6 was 50-100pg/ml, while MCP-1 was significantly enhanced by high glucose and palmitic acid (PA) stimulation, and the protein concentration reached 1500pg/ml. Hypertrophied adipocytes have been found to secrete large amounts of MCP-1, which function as a chemoattractant that enhances macrophage infiltration into adipose tissue in obese mice and humans.(Engin 2017) Infect, MCP-1 is secreted by adipocytes in crown-like structures, and stimulate

the proliferation of surrounding adipose tissue macrophages (ATMs). (Amano et al. 2014) MCP-1 secreted by adipose tissue contributes to the macrophage infiltration in diet-induced obese and insulin resistance animals. (Kanda et al. 2006) After acute high saturated fatty acid stimulation preadipocytes have a heightened inflammatory cytokine response, especially by the MCP-1 expression. Furthermore, preadipocytes recruit macrophage via MCP-1 in adipose tissue and produce inflammatory responses. (Dommel and Bluher 2021; Dordevic, Konstantopoulos, and Cameron-Smith 2014) The increased production of MCP-1 by adipocytes might contribute to a pro-inflammatory state. However, our results suggest that GPR50 significantly inhibit the secretion of MCP-1 and alleviate the pro-inflammatory state of adipocytes. In recent years, there has been increasing evidence that inflammation is an indispensable cause of IR during the development of obesity and T2DM. Studies in obesity and T2DM have revealed the correlation of the pro-inflammatory signaling pathways and insulin sensitivity. (Feve and Bastard 2009; Zheng and Zheng 2016; Catrysse and van Loo 2017) Some GPCRs were shown to participate critically in certain processes of inflammation., such as GPR84 and GPR91, both of them play an important regulatory role in inflammation on immune cells. (Nagasaki et al. 2012; Miyamoto et al. 2017; Littlewood-Evans et al. 2016; Rubic-Schneider et al. 2017) However, there are still many unknown mediators which are involved in regulating inflammation in adipocyte. Finding the new mediators associated with adipocyte low-grade inflammation, sufficient to induce IR in obesity and T2DM individuals, is an important work to do.

From this study, it seems that GPR50 has inhibitory effect on inflammation in adipocyte 3T3-L1, and that chronic tissue inflammation is an important cause of IR induced by obesity. (Soedono and Cho 2021) Therefore, we investigated the effects of GPR50 on insulin signaling pathways. Insulin receptor activation contributes to IRS1 phosphorylation, which initiates downstream signaling. When IRS1 is phosphorylated, its downstream signaling capacity is decreased. (Yang, Vijayakumar, and Kahn 2018; Copps and White 2012) According to our experimental results, GPR50 deficiency may significantly activate insulin signaling pathway in which IRS1 involved. As shown in Fig. 5, p-IRS1 was significantly triggered in the GPR50-deficient group relative to the control group. Interestingly, although the high glucose and PA treatment group reduced p-IRS1 expression by nearly 30 percent, high glucose and PA treatment did not downregulate p-IRS1 expression in GPR50 knockout cells as it did in 3T3-L1 cells. These results indicated that GPR50 can effectively regulate IRS1 phosphorylation, whereas stimulation by high glucose and PA could regulate IRS-1 through GPR50 directly. And we also found that GPR50 deficiency activated p-AKT, although stimulation of high glucose and PA activated p-AKT strongly, in GPR50 knockout 3T3-L1 cells, p-AKT was activated further. These results indicated that GPR50 might mediate the p-IRS1/AKT pathway.

PPAR- γ regulates multiple genes transcription involved in the adipose precursor cell differentiation, regulates glucose uptake mediated by insulin, and increases insulin sensitivity. PPAR- γ is closely related to inflammation and insulin resistance. (Boughanem et al. 2019) PPAR- γ is important in regulating the of inflammatory cytokines. Downregulation of PPAR- γ could strongly contribute to these effects of inflammatory cytokines on adipocytes. Also, by modulating the IRS-1 expressions, PPAR- γ plays an important role in the insulin signaling pathway. (Ahmadian et al. 2013) PPAR- γ agonist Fenofibrate and Saroglitazar can improves IR. (Feng et al. 2017; Kumar et al. 2020) Induction of PPAR- γ expression in adipocytes decreased IR in obese mice in a previous study. (Soares et al. 2013)

In our study, we found that knout out GPR50 significant inhibit PPAR- γ expression, this result suggests that GPR50 is conducive to the activation of PPAR- γ with increased plasma levels of free fatty acids (FFA). PPAR- γ activation in adipose tissue promotes lipid uptake and storage through induction of target genes include aP2, LPL, CD36 and so on. PPAR- γ also influences the effects of inflammatory cytokines and adipokines, including adiponectin, MCP-1, and resistin. As well as modulating the expression of adipokines, these changes can have beneficial effects on systemic glucose metabolism, including suppressing hepatic glucose uptake and stimulating skeletal muscle glucose uptake. Considering the role inflammation, insulin signaling pathway and PPAR- γ expression play in the pathogenesis of insulin resistance, we tentatively believe GPR50 is involved in regulating inflammation and insulin signaling, therefore, it may contribute positively to insulin resistance.

During our experiments, we found GPR50 may affect 3T3-L1 differentiation (Figure.A2 A, B unpublished data). After 12 days of lipogenic differentiation in GPR50-deficient 3T3L1 cell lines, intracellular lipid droplet accumulation was significantly less

than to the control group. As we know, several GPCR activation in adipose tissue were linked to adipocyte function.(Chang et al. 2013) Such as GPR43 can promote 3T3-L1 cell differentiation and lipid droplet accumulation.(Neuhofer et al. 2013) And we know that lipogenic differentiation process affects PPAR- γ expression. So, we detected the expression of PPAR- γ and HSL (Figure.A2 C, D) (hormone-sensitive lipase, HSL) in mRNA level. Although, we found GPR50 can affect lipogenic differentiation, but we are not yet able to clarify what and how GPR50 plays a role in lipogenic differentiation, and further experiments are needed to demonstrate that GPR50 can indeed influence lipogenic differentiation and its effect on obesity-T2DM.

Conclusion

We first identified GPR50 as a novel IR target candidate in adipose tissue. We demonstrate that GPR50 can improve high glucose and palmitic acid induced adipose tissue inflammation and may be able to inhibit insulin signaling by regulating the phosphorylation of IRS-1 and AKT. Expression of PPAR- γ transcription factor was also enhanced. Collectively, the above results suggest that GPR50 ameliorate inflammation and is involved in insulin signaling might via promote PPAR- γ expression, but there're still many details need to be elucidated (Figure 8).

Abbreviations

T2DM, type 2 diabetes mellitus; HFD, high-fat diet; IR, insulin resistance; PA, palmitic acid; IRS, insulin receptor substrate; MCP-1, monocyte chemoattractant protein 1; PI3K, PPAR- γ , phosphatidylinositol 3-kinase; peroxisome proliferators-activated receptor- γ ; FFA, free fatty acids; HSL, hormone-sensitive lipase.

Declarations

Data Sharing Statement

Microarray and sample annotation data were deposited in Gene Expression Omnibus under accession number GSE100028. Direct link to the deposited data is available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE100028>. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

All the procedures were under the supervision and approved by the Ethics Committee for Animal Research, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences (Approval Number: SIAT-IRB-170401-YGS-RPG-A0312-01 and date of approval is June 2017).

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Disclosure

Zhenyu Yao, Chang Liu, Jun Meng, Jing long, Long Li, Weicong Qiu, Cairong Li, Jian V. Zhang, Pei-Gen Ren declare that there is no conflict of interests regarding the publication of this article.

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Tables

Table 1 is not available with this version

Figures

Figure 1

Hierarchical clustering analysis of most 20 differentially expressed genes in chow mice and HFD mice. Differentially expressed mRNAs in CHOW (n=3 from 5) and HFD mice (n=3 from 5). Red and blue color indicates up-regulated and down-regulated transcripts, respectively.

Figure 2

Analysis of GPR50 expression in mouse adipose tissue and 3T3-L1 cell line. Epididymal fat pads of CHOW and HFD C57BL/6J mice were lysed, GPR50 mRNA expression detected by Q-PCR (A) protein expression of GPR50 evaluated by Western blot (B-C). 3T3-L1 pre-adipocyte differentiated into adipocytes for more than 6 days, GPR50 mRNA expression was detected by Q-PCR (D) and GPR50 protein level was evaluated by Western blot (E-F). Western blots are shown along with relative densities, determined using Image J software. Results are represented of three independent experiments. Values in bar graphs are means $\pm$ SEM. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ when compared with day 0.

Figure 3

Analysis of GPR50 expression in GPR50 knockout 3T3-L1 cell line. Protein level expression of GPR50 was evaluated by Western blot in GPR50 knockout 3T3-L1 cell line (A-B). Western blots are shown along with relative densities, determined using Image J software. Results are represented of three independent experiments. Values in bar graphs are means $\pm$ SEM. ***: $P < 0.001$ when compared with control.

Figure 4

GPR50 inhibits inflammation in GPR50 knockout 3T3L1 cell line. Two cell lines stimulated with high glucose (16.7mM) and PA (0.25mM) for 48 hours. The relative mRNA expressions of GPR50 (A), IL-6 (B), IL-1 β (C), and MCP-1 (D) were analyzed by Q-PCR. IL-6 (E), IL-1 β (F), and MCP-1 (G) were analyzed by Elisa. Data were analyzed using two-way ANOVA. Results are represented of three independent experiments. Values in bar graphs are means $\pm$ SEM. ***: $p < 0.0001$ compared to Control, ####: $p < 0.0001$ compared to Control + Glucose,

: $p < 0.0001$ compared to Control + PA.

Figure 5

Deficiency of GPR50 activated insulin signaling pathway in 3T3-L1 cell line. The proteins expression of p-IRS-1, IRS-1, p-Akt, Akt and GAPDH were analyzed by western blot (A). Relative expression of p-IRS-1/IRS-1(long exposure) (B) and p-Akt/Akt (C) representative. Western blots are shown along with relative densities, determined using Image J software. Data were analyzed using two-way ANOVA. Results are represented of three independent experiments. Values in bar graphs are means $\pm$ SEM. *, ****: $p < 0.05$, $p < 0.0001$ compared to Control; ###, ####: $p < 0.001$, $p < 0.0001$ compared to Control + Glucose, , $p < 0.0001$ compared to Control + PA. &&&&, $p < 0.0001$ compared to GPR50 knockout.

Figure 6

Deficiency of GPR50 suppressed PPAR- γ expression in 3T3-L1 cell line. The mRNA level expression of PPAR- γ were analyzed by Q-PCR (A). The protein level of PPAR- γ was analyzed by Western blot (B-C). Western blots are shown along with relative densities, determined using Image J software. Data were analyzed using two-way ANOVA. Results are represented of three independent experiments. Values in bar graphs are means $\pm$ SEM. ****: $p < 0.0001$ compared to Control, ####: $p < 0.0001$ compared to Control + Glucose,

: $p < 0.0001$ compared to Control + PA.

Figure 7

Deficiency of GPR50 activated insulin signaling pathway in 3T3-L1 cell line with 100 nM insulin stimulate. The proteins expression of p-IRS-1(Tyr612), IRS-1, p-Akt (Ser473), Akt and β-actin were analyzed by western blot (A-D). Relative expression of p-IRS-1/IRS-1, p-Akt/Akt and PPAR-γ/ β-actin representative. Western blots are shown along with relative densities, determined using Image J software. Data were analyzed using two-way ANOVA. Values in bar graphs are means ± SEM. **, ****: $p < 0.01$ and $p < 0.0001$ compared to Control; ####: $p < 0.0001$ compared to Control + insulin; \$,

: $p < 0.05$, $p < 0.0001$ compared to GPR50 knockout.

Figure 8

Speculated working model for GPR50-mediated inflammation and insulin signaling regulation in 3T3-L1 cell line. GPR50 inhibits inflammation in adipose tissue and improves insulin resistance induced by high glucose or palmitic acid by inhibiting phosphorylation of IRS-1 and AKT. Together, GPR50 might improve inflammation and insulin signaling and promote PPAR-γ expression.

Supplementary Files

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