Cell. Mol. Biol. 58 (Supp): OL1757-OL1762
Published on October 8, 2012
DOI 10.1170/206

Cellular & Molecular Biology
http://www.cellmolbiol.com

G-PROTEIN COUPLED RECEPTOR 120 IS INVOLVED IN GLUCOSE METABOLISM IN FAT CELLS

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Abstract

Free fatty acids (FFA) are closely related to insulin resistance in which proteins such as insulin receptor substrate 1 (IRS-1), phosphatidylinositol 3 kinase (PI3K) and glucose transporter type 4 (GLUT4) are involved. Recent researches have shown that G-protein coupled receptor 120 (GPR120) is a receptor for medium and long chain FFA. This study aimed to evaluate the relationship between GPR120 and proteins related to the glucose metabolism. We used siRNA technique to down-regulate the GPR120 expression in 3T3-L1 cells before incubation with palmitic acid (PA), and evaluated the effect of GPR120 expression on the levels of IRS-1, PI3K and GLUT4 after PA induction. RT-PCR and western blot were performed to detect gene and protein levels in differentiated cells. RT-PCR result showed that GPR120 mRNA significantly increased in differentiated cells. GPR120-SiRNA transfection significantly down-regulated GPR120 gene and protein level in differentiated 3T3-L1 cells on day 5 after PA-induced and also decreased lipid droplets accumulated within the cells. SiRNA-mediated decreased of GRP120 was associated with significant reductions in gene and protein levels of IRS-1 and GLUT4. Our results showed that the expression level of GPR120 affected the expression of proteins related to the glucose metabolism, suggesting a contribution of GPR120 in the insulin resistance.

Key words:Free fatty acids**,** insulin resistance**,** G-protein coupled receptor 120, GPR120, glucose metabolism.

Article information

Received on March 3, 2012
Accepted on September 21, 2012

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INTRODUCTION

Free fatty acids (FFA) are precursors of cellular signal substance, and are important to the biological functions of the cell. Recent studies have found that insulin sensitivity is directly related to the FFA levels in serum and tissues. FFA can substantially inhibit the phosphorylation level of the tyrosine residues on the insulin receptor (InsR) and the insulin receptor substrate 1 (IRS-1) in liver, skeletal muscle and fatty tissues, hence interfere the normal transduction of insulin signals and participate in insulin resistance (IR) (13). In mammals insulin plays a key role in regulating the metabolisms in liver, skeletal muscle and fat cells (1, 2) and many proteins have been shown important roles in glucose metabolism and insulin signaling. Large docking protein IRS-1, the main substrate for insulin and other protein tyrosine kinases, mediates the activities of insulin, including adhering and activating PI3K, and then promoting glucose transportation. However, the underlying mechanism of this process is yet not fully elucidated.

More recently it has been shown that several G-protein-coupled receptors (GPCR) are receptors for FFA. One member, G-protein coupled receptor 120 (GPR120), is expressed in the fatty tissues of both human and mouse (7) and can be activated by saturated FFAs of 14–18 carbon atoms (C14–18) or unsaturated FFAs of 16–22 carbon atoms (C16–22) (8). The expression level of GPR120 mRNA is closely related to the differentiation of 3T3-L1 cells (7). A study has shown that both *in vivo* and *in vitro*, stimulating GPR120 can increase glucagon-like peptide-1 (GLP-1) level, and thus increase the insulin level in the circulation (15). GPR120 activation in 3T3-L1 cells can promote the transfer of glucose transporter type 4 (GLUT4) to the cell membrane (14). Therefore the relationship between the fatty acid receptor GPR120 in fatty tissues and the insulin signal pathway has become a new research target.

Palmitic acid (PA) is a saturated fatty acid of 16 carbon atoms and a ligand of GPR120. Incubation with 5 mM PA can induce insulin resistance (18) and it is associated with expressions of IRS-I (17) and GLUT4 (16). In this study, we used PA to induce 3T3-L1 cell differentiation. Using this model, we down-regulate the GPR120 expression in 3T3-L1 cells using siRNA technique and to monitor the fatty-acid-induced changes in the expression of genes and proteins related to glucose metabolism, which affects insulin signal pathway.

MATERIALS AND METHODS

Synthesis of siRNA

Fluorescently labeled siRNA (GPR120-siRNA) targeting mouse GPR120 mRNA (NM_181748.2) and the negative control siRNA (NC-siRNA) were chemically synthesized by Shanghai GenePharma Co. The GPR120-siRNA targeted at mRNA sites 1116-1134.

The siRNA sequences were as follows: 1) GPR120-siRNA, sense: 5'-GUGCAUGUAAAGGGAGUUATT-3', antisense: 5'-UAACUCCCUUUACAUGCACTT-3'; 2) NC-siRNA, sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'.

3T3-L1 cell culture and siRNA transfection

The 3T3-L1 cells were purchased from the Chinese Academy of Sciences Cell Bank. Cells were cultured in DMEM medium containing 10% fetal calf serum in the

incubator containing 5% CO_2 at 37°C. When the cells reached 70%-80% confluence, siRNA-GPR120 or NC-siRNA were transfected into the cells using X-treme-GENE siRNA Transfection Reagent (Roche). In both cases, 40 μg siRNAs were dissolved in 160 μl DEPC water and the transfection was performed according to the manufacture instruction. After 24 h, the culture medium was changed to induction medium I (DMEM containing 10% FBS, 0.5 mmol/L isobutylmethylxanthine (IBMX, Sigma), 10 μg/ml insulin (Sigma), and 1.0 μmol/L dexamethasone (Dex, Sigma)) and the time was marked as day 0. On day 2, the medium was switched to induction medium II (DMEM+10μg/ml insulin+10% FBS). On day 4, the medium was switched to DMEM medium containing 10% FBS and 0.5 mM palmitic acid (PA, Sigma) and the culture was maintained for 24 h. Then insulin was added to reach a final concentration of 100 nM. On day 5, the medium was switched to DMEM medium containing 10% FBS, and was changed every other day afterwards. On day 5, 3T3-L1 RNAs were extracted and the effect of transfection was evaluated by PCR.

Semi-quantitative RT-PCR and real time PCR

To detect SiRNA knock-down of GPR120, the RNAs were extracted from 3T3-L1 cells on the 5th day after induction using extract kit (Omega), and the cDNAs were obtained via reverse transcription (TaKaRa PrimeScript™ RT-PCR Kit) for PCR amplification. Primer sequences were as follows: GPR120: F: 5'-GATTGGCCCAACCG-CATAG-3' R: 5'-CTCGGATCTGGTGGCTCTCA-3'. The reaction conditions were as follows: 94°C, 30 sec for denaturation; 58°C, 1 min for annealing; 40 cycles of extension at 72°C, 1 min; 72°C, 1min for the last cycle of extension. The products were stored at 4°C. Next 2% agarose gel was made and RT-PCR products mixed with buffer were loaded into the wells, subjected to electrophoresis under 140 V for 40min. The gel was then covered and stained with ethidium bromide (1 μg/μl) for 5min before observed and recorded by automated electrophoresis gel image analyzer. The densities of the electrophoretic bands were measured with β-actin used as the internal reference; the results were presented as the percentage ratio of GRR120 band density to β-actin band density.

To compare the level of gene expression of PI3K, IRS-1 and GLUT4, real-time PCR was performed under the instruction of LightCycler480 SYBR Green I Master (Roche). The primers of different genes were as follows: β-actin: F: 5'-ACCAACTGGGACGATATGGAGAAGA-3';R: 5'-TACGACCAGAGGCATACAGGGACAA-3'; PI3K, F: 5'-GAGCAAAGCCAAGGAAACTG-3', R: 5'-TCTCCCCAGTACCATTCAGC-3'; IRS-1 and GLUT4 primers used were as described by DO (3).

Western blot

After the 3T3-L1 cells were transfected, induced and incubated with PA, the cells were collected on the 5th day and subjected to SDS-PAGE (15% separation gel and 5% spacer gel). Electrophoretic transfer was then performed at 100 mA for 60 min. Next 5% skim milk was used for blocking for 20 min, followed by 15ml PBST wash for 5min. GPR120 antibody, IRS-1 antibody, phosphatidylinositol 3 kinase (PI3K) p85 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GLUT4 antibodies (R&D Systems, USA) and β-actin antibodies (Lab Vision, USA) were diluted by 1:1000 and processed with Western blot enhancer (M2501, HaiGene, Harbin, China) for 2 h. The primary antibodies were then pre-processed with 10ml Rapid WB buffer for 40 min before a 5 min PBST wash. HRP-labeled secondary antibody diluted by 1:10000 was added together with 10ml Rapid WB buffer for a 20 min-incubation. After secondary antibody reaction, the gel was washed three times with PBST, 5min each. Two milliliter ECL reagent (HaiGene, M2301) was used for dark room exposure, and the exposure time was 30 sec.

Oil red O staining

Eight days after the 3T3-L1 cell induction following siRNA transfection, the cells were washed with PBS for three times, fixed in 10% formalin for 30 min, and followed by PBS wash for two times. Then the cells were stained with Oil red O working solution (0.5 g Oil red O powder dissolved in 100 ml 98% isopropanol) for 8 min, followed by color separation with 60% isopropanol for 10-20 sec, and rinsed with distilled water before observation under the microscope.

Statistical analysis

All experiments were repeated three times. All data were presented as means ± SD. t-Test was performed with JMP4.0 (SAS Institute Inc., Cary, NC, USA) for two-group comparison and one-way ANOVA was performed for multi-group comparison followed by Turkey post hoc test. Results were considered statistically significant at $P<0.05$.

RESULTS

Differentiation induction increased GPR120 mRNA in 3T3-L1 cells

To study the association between GPR120 and 3T3-L1 cell differentiation, we compared the mRNA level of GPR120 in non-differentiated 3T3-L1 cells and differentiated 3T3-L1 cells induced by IBMX+Dex+insulin. RT-PCR result showed that there was no GPR120 expression in non-differentiated 3T3-L1 cells, whereas GPR120 gene expression was significantly increased in 3T3-L1 cells on the 5th day after differentiation induction (Fig. 1). This suggested that 3T3-L1 differentiation was associated with upregulation of GPR120 expression.

GPR120-SiRNA decreased GPR120 mRNA and protein expression in differentiated 3T3-L1 cells

Fluorescent image showed that 3T3-L1 cells transfected with fluorescently labeled siRNA were strongly positive with green fluorescence at 24 h after transfection, suggesting an efficient siRNA transfection into the cells (Fig. 2 A).

We further used RT-PCR and WB detected GPR120 mRNA and protein expression level in differentiated 3T3-L1 cells on day 5 after induction. GPR120-SiRNA transfection significantly know-down GPR120 gene (Fig. 2 B) and protein (Fig. 2 C) expression in differentiated 3T3-L1 cells compared to NC-SiRNA transfection.

GPR120-SiRNA transfection decreased lipid droplets in differentiated 3T3-L1 cells

The differentiation level of 3T3-L1 cells was determined by Oil red O staining on day 8 after induction. 3T3-L1 cells transfected with NC-siRNA showed numerous

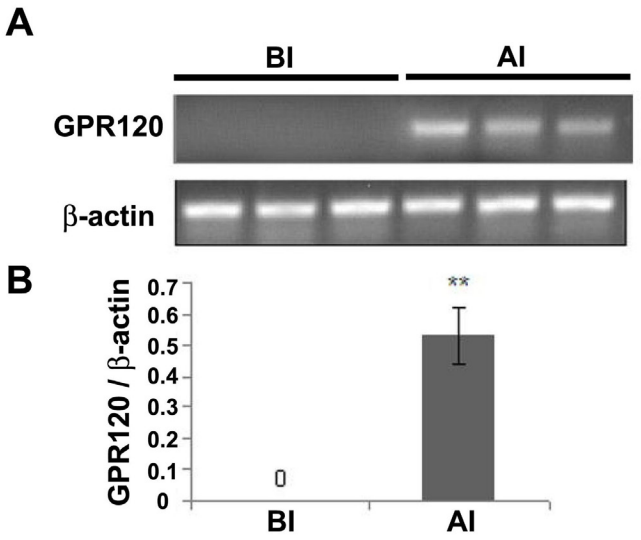

Figure 1. The mRNA level of GPR120 increased in differentiated 3T3-L1 cells. (A) RT-PCR result. (B) Quantification of RT-PCR. ** p<0.01

red lipid droplets around the nuclei (arrows in Fig 3A), with some small lipid droplets fused into bigger ones. In contrast, 3T3-L1 cells transfected with GPR120-siRNA showed noticeably less number and smaller size of red lipid droplets (Fig 3B), with lipid droplets also arranged around the nuclei. This result indicated that decrease GPR120 expression using siRNA was associated with attenuated differentiation level of 3T3-L1 cells in response to IBMX+Dex+insulin induction, suggesting an involvement of GPR120 in 3T3-L1 cell differentiation.

The effect of GPR120 expression on the levels of IRS-1, PI3K and GLUT4 after PA induction

To further study the effect of GPR120 expression on the levels of proteins involved in glucose metabolism, we used RT-PCR and WT to detect gene and protein levels of IRS-1, PI3K and GLUT4 on day 5 after PA induction. Compared to NC-SiRNA transfection, GPR120-SiRNA transfection significantly decreased gene expressions of IRS-1 (0.794±0.024 v.s. 1.003±0.115, p=0.031) and GLUT4

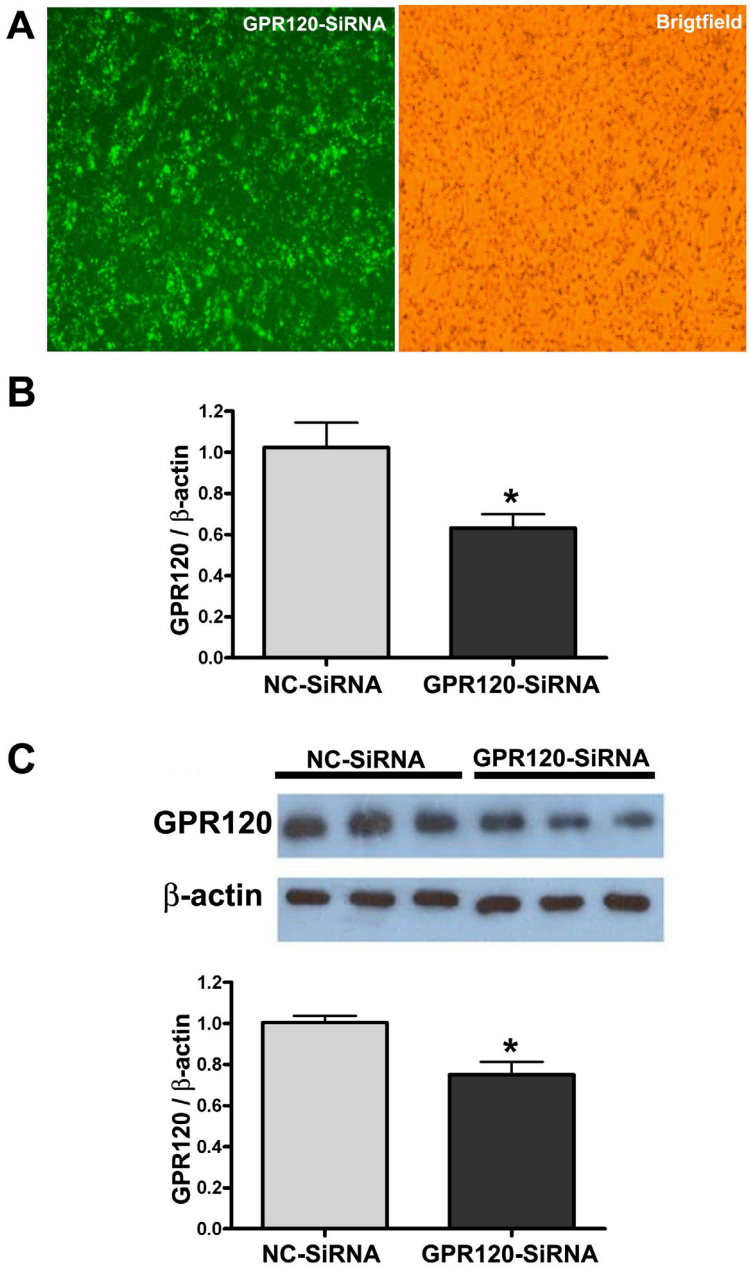

Figure 2. GPR120 SiRNA transfection down-regulated gene and protein level of GPR120 in differentiated 3T3-L1 cells. (A) Fluorescent SiRNA transfection showed high transduction efficiency, 50 x magnification. (B) RT-PCR detection of GPR120 mRNA. (C) WB for GPR120 protein. *p<0.05.

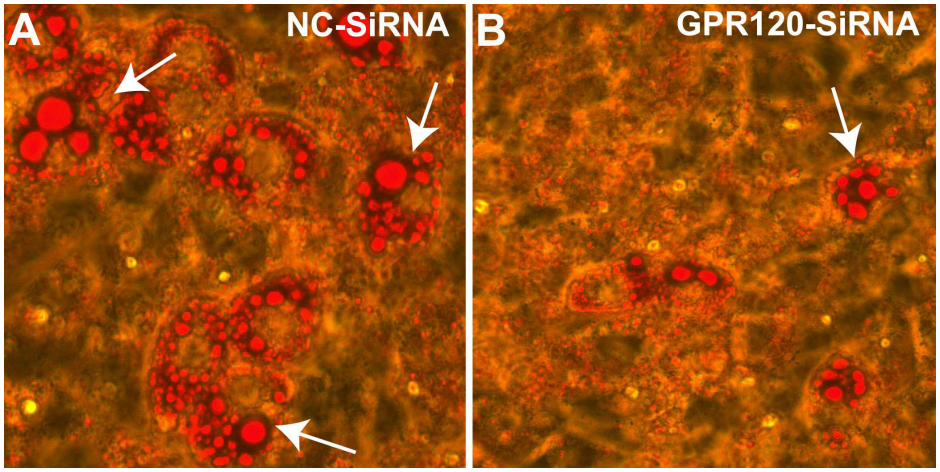

Figure 3. Oil red O staining for differentiated 3T3-L1 cells. (A) NC-SiRNA, (B) GPR120-SiRNA. White arrows indicated red lipid droplets. 200X magnification.

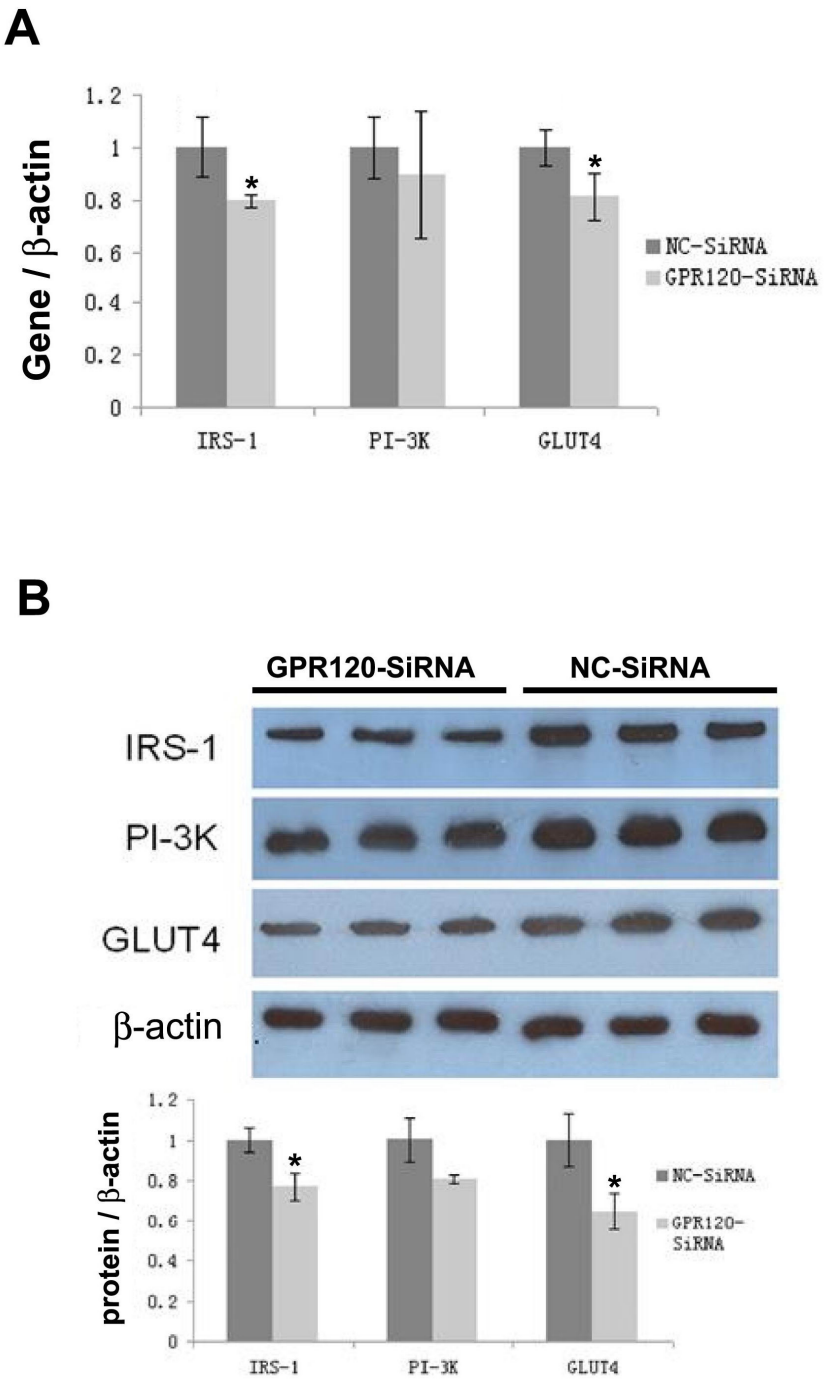

Figure 4. Gene and protein expressions of IRS, PI-3k and GLUT4 in 3T3-L1 cells transfected with GPR120-SiRNA on day 5 after induction. (A) Quantification of real-time PCR results. (B) WB for protein level of IRS, PI-3k and GLUT4. *p<0.05.

(0.810±0.088 v.s. 1.001±0.086, p=0.030). No significant change was found in PI-3K gene expression in GPR120-SiRNA cells (0.895±0.241 v.s. 1.005±0.1194, p=0.747). Down-regulation of GPR120-SiRNA reduced protein levels of IRS-1 (0.769±0.067 v.s. 0.990±0.059, p<0.05) and GLUT4 (0.645±0.088 v.s. 1.001±0.129, p<0.05). Protein level of PI-3K showed a trend of but not significant decrease (0.803±0.021 v.s. 1.001±0.112, p=0.059) in differentiated 3T3-L1 cells with GRP120 knock-down (Fig. 4 A and B).

DISCUSSION

As a receptor for long chain FFAs, GPR120 may play an important role in mediating FFA signals and changing the differentiation and glucolipid metabolism of fat cells. Our results showed that GPR120 was not expressed in 3T3-L1 cells before induction, but the GPR120 expression was detectable 5 days after induction, suggesting that GPR120 is only expressed after 3T3-L1 cell differentiation occurs. When GPR120 expression decreased, the number and volume of lipid droplets stained by Oil red O in the 3T3-L1 cells on day 8 after induction were both substantially decreased. This suggests that GPR120 is also involved in processes that affect the adipocyte differentiation and metabolism of 3T3-L1 cells.

In this study, we observed a decreased level of IRS-1 in 3T3-L1 cells with GRP120 knock-down, suggesting that GRP120 expression level affected IRS-1 level. IRS-1 is the main substrate for insulin and it can promote glucose transportation through activating PI3K. Studies have shown that normal level of IRS-1 in tissue cells is the basis for normal insulin signaling. When IRS-1 concentration drops to certain degree, the intracellular transduction of insulin signal is blocked (17). This suggests that down-regulation GRP120 expression resulted in decreased IRS-1 level therefore affecting activation of insulin signaling pathway.

By controlling the shift of specific glucose transporter GLUT4 from intracellular vesicles to cellular surface, insulin regulates the transportation of glucose to skeletal muscle and fat cells (4, 9, 10). The expression of GLUT4 is rigorously controlled, and factors that can influence its expression and dislocation may cause abnormality in the transmembrane transport of glucose, in turn leading to insulin resistance. A study has shown that the glucose tolerance of GPR120 knock-out mice is mildly impaired compared to wild mice (12), whereas the insulin secretion of GPR120 knock-out mice is twice that of wild mice. This hyperinsulinemia along with mild glucose tolerance impairment suggests the occurrence of insulin resistance. In this study, both gene and protein level of GLU4 were significantly decreased in GPR120 knock-down cells. GPR120 expression might contribute to insulin resistance through affecting level of GLU4 and glucose transportation.

The PA used in this study is a saturated fatty acid of 16 carbon atoms and a ligand of GPR120. Ping et al. (18) reported that incubation of 5 mM PA with fat cells induced insulin resistance. When insulin resistance occurs, both IRS-I (17) and GLUT4 (16) expressions significantly decrease. Gao et al. (5) have shown that high concentration of FFAs can increase the serine phosphorylation and decrease the tyrosine phosphorylation of IRS-1, and thus induce insulin resistance. Goodyear et al. (6) have also reported that the IRS-1 expression level in the skeletal muscle cells of obese patients is 54% lower than normal subjects. In this experiment, after the GPR120 expression in 3T3-L1 cells was down-regulated using the siRNA technique, the expression levels of key proteins in the insulin signal pathway of 3T3-L1 cells IRS-1 and GLUT4 were significantly reduced under high PA stimulation compared to negative control group. It suggests that when stimulated by PA, fat cells with GPR120 down-regulation are more susceptible to insulin resistance, or exhibit more serious insulin resistance when compared to negative controls.

In our study we did not observe significant PA-induced PI3K decrease in 3T3-L1 cells when GPR120 expression was down-regulated. Our explanation is that the change in the activity rather than the change in the level of PI3K leads to changes in glucose transport mediated by insulin signaling. This phenomenon might need further investigation in the future study. There were some limitations in our study. We did not look at the phosphorylation mechanisms involved in the signaling pathways. For example, we did not investigate the GPR120-siRNA effect on the AKT phosphorylation, mTOR activation and protein synthesis when the cells were treated with palmitic acid. It has been demonstrated that GPR120 activation by FFA induces an anti-apoptotic process via AKT-PI3K and ERK activation (11). It would be of great interest to know if the GPR120 is involved in apoptotic process in 3T3-L1 cells. We did not look at the effect of siRNA on the GLUT4 translocation and transport to the plasma membrane once there was a decrease of GLUT4 expression in transfected cells. All these points will be addressed in our future studies.

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