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Role of Peroxisome Proliferator-Activated Receptor Gamma in Glucose-induced Insulin Secretion

Ze-Kuan XU[1,2], Neng-Guin CHEN[1], Chang-Yan MA[1], Zhuo-Xian MENG[1], Yu-Jie SUN[1], and Xiao HAN[1]*

[1] *Key Laboratory of Human Functional Genomics of Jiangsu Province, Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing 210029, China;*
[2] *Department of Normal Surgery, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China*

Abstract Peroxisome proliferator-activated receptor (PPAR) isoforms (α and γ) are known to be expressed in pancreatic islets as well as in insulin-producing cell lines. Ligands of PPAR have been shown to enhance glucose-induced insulin secretion in rat pancreatic islets. However, their effect on insulin secretion is still unclear. To understand the molecular mechanism by which PPARγ exerts its effect on glucose-induced insulin secretion, we examined the endogenous activity of PPAR isoforms, and studied the PPARγ function and its target gene expression in INS-1 cells. We found that: (1) endogenous PPARγ was activated in a ligand-dependent manner in INS-1 cells; (2) overexpression of PPARγ in the absence of PPARγ ligands enhanced glucose-induced insulin secretion, which indicates that the increased glucose-induced insulin secretion is a PPARγ-mediated event; (3) the addition of both PPARγ and retinoid X receptor (RXR) ligands showed a synergistic effect on the augmentation of reporter activity, suggesting that the hetero-dimerization of PPARγ and RXR is required for the regulation of the target genes; (4) PPARs upregulated both the glucose transporter 2 (GLUT2) and Cb1-associated protein (CAP) genes in INS-1 cells. Our findings suggest an important mechanistic pathway in which PPARγ enhances glucose-induced insulin secretion by activating the expression of GLUT2 and CAP genes in a ligand-dependent manner.

Key words PPARγ; ligand; glucose-induced insulin secretion; glucose transporter 2; Cb1-associated protein

The peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors that regulate gene networks involved in cellular development, differentiation and metabolism [1]. PPARs exist in three forms in rat: PPARα, γ and δ, which are members of the nuclear hormone receptor superfamily. PPARs heterodimerize with retinoid X receptor (RXR) in order to bind to DNA recognition sequences, which contain a direct repeat core-site separated by one nucleotide (*NNN*-AGGTCA-*N*-AGGTCA). These complexes destabilize chromatin and activate transcription [2,3]. Through this mechanism, PPARs directly regulate transcription in response to their specific ligands. In addition to ligand-dependent transcriptional activation, PPARγ activity is also regulated by mitogen-activated protein (MAP) kinase [4] or c-Jun N-terminal kinase signaling pathways [5]. Phosphorylation of PPARγ at a consensus MAP kinase site inhibits the ligand-independent and ligand-dependent transactivation functions [6]. These findings provide an important mechanism for cross-talk between PPARγ and other cellular signaling pathways in a physiological context [7].

Recently, PPARs have been shown to be involved in diabetes, cancer and inflammatory diseases. The thiazolidinedione (TZD) class of antidiabetic drugs alleviates insulin resistance and hyperglycemia in human diabetes [8,9]. Several antidiabetic agents in the TZD class such as rosiglitazone, troglitazone and pioglitazone, have been identified as ligands of PPARγ [10–13]. There is evidence that the effect of TZD on increased insulin sensitivity is mediated through PPARγ [14,15]. PPARγ

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*Corresponding author: Tel, 86-25-86862731; Fax, 86-25-86862731; E-mail, hanxiao@njmu.edu.cn

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ligands are shown to augment glucose disposal in peripheral tissues by increasing expression of the glucose transporter genes glucose transporter 1 (GLUT1) and GLUT4 [16]. Several clinical studies linked the mutation in different regions of PPARγ with insulin resistance, diabetes and hypertension [17–21]. It is generally accepted that PPARγ increases glucose transport activity and transporter expression in adipose tissues and muscles.

In pancreatic islet, GLUT2 was reported to act as a glucose sensor [22]. Pancreatic islets treated with troglitazone increased the expression level of GLUT2 in Zucker Diabetic Fatty rats [23]. A functional PPAR response element (PPRE) has been identified in the rat GLUT2 gene promoter and it is suggested that PPARs may be involved in the regulation of glucose-induced insulin secretion [24]. However, the direct correlation between PPARs and glucose-induced insulin secretion needs to be established.

This work is designed to investigate the molecular role of PPARα and γ in glucose-induced insulin secretion and to focus on the function and target genes of PPARγ.

Materials and Methods

Reagents

Rosiglitazone (BRL 49653) was obtained from Biomol (Plymouth Meeting, USA). Wy 14643 was purchased from Cayman Chemical (Ann Arbor, USA). 9-cis-retinoic acid was obtained from Sigma (St. Louis, USA). Cell culture reagents were from Invitrogen (Carlsbad, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, USA). Expression plasmids pCMX-mPPARα, pCMX-mPPARγ and pCMX-VP-mPPARγ were modified by the cDNA constructs obtained from Invitrogen. PPRE3-TK-Luc reporter construct was made as previously described [25]. pCMX-mPPARγ-S84A was constructed according to published procedures [6].

Cell culture

INS-1 cells, a widely used rat insulinoma β cell line for insulin secretion studies [26], were from Dr. HAN (Diabetes and Genetics Research Center, City of Hope National Medical Center, Duarte, USA) and were cultured to near 100% confluence in RPMI 1640 medium supplemented with 11 mM *D*-glucose, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM *L*-glutamine, 1 mM sodium pyruvate and 50 μM-mercaptoethanol. All tissue cultures were performed in a Forma Scientific tissue culture incubator that provided an environment of 5% CO_2.

Transient transfection and luciferase assay

One day before transfection, INS-1 cells were dispersed with trypsin-EDTA solution and counted. The cells were seeded into 12-well plates at a density of 2×10^5 per well to attain 90% confluence the next day. PPRE3-TK-Luc, a reporter construct containing PPRE (0.8 μg/well) and β-gal plasmid (0.4 μg/well, Clontech, Palo Alto, USA) were incubated with Lipofectamine 2000 reagent (Gibco, Grand Island, USA) for 30 min at room temperature. The cell culture medium was removed and replaced with 1 ml of RPMI 1640 containing 10% FBS and the lipid/DNA complex, and the cells were cultured for 18 h. Then the medium was changed to phenol red-free RPMI 1640 with 5% stripped-FBS (FBS deprived of the growth factors by charcoal) and the cells were incubated for an additional 18 h. Then ligands were added, and the cells were harvested after a further 18 h of incubation. In addition, we followed a similar transfection procedure with the pEGFP-N1 (Clontech) and counted the enhanced green fluorescent protein-positive cells versus the total cell number in order to estimate the transfection efficiency, which was approximately 75% here. The luciferase activity was measured with a TD-20/20 luminometer (Turner Designs, Sunnyvale, USA), using 100 μl of whole cell lysate and the same volume of luciferase assay reagent (Promega, Madison, USA). An aliquot of the same cell lysate for each sample was used to measure β-galactosidase activity to normalize luciferase activity. Luciferase assays were performed in triplicate and repeated four times.

RNA extraction, real-time quantitative polymerase chain reaction (RT-QPCR) and insulin secretion measurement

Cells were transfected with various expression vectors overnight. Culture plates were washed with 1×PBS and then treated with ligands in RPMI 1640 (11 mM glucose and 10% stripped-FBS) for 24 h. To stabilize the insulin secretion, the transfected cells were incubated in RPMI 1640 (3 mM glucose and 0.1% bovine serum albumin) for 1 h, then the supernatant was removed and the cells were incubated with the same medium for 2 h, at the time point, the supernatant was collected for later insulin measurement. The glucose concentration in the medium was then increased to 20 mM and the transfected cells were incubated for an additional 2 h. Supernatant was frozen at −70 °C and insulin determination assay was performed later. The amount of insulin in the supernatant was detected

by rat insulin ELISA kit (Crystal Chem, Chicago, USA). Total RNA was isolated from cells by RNeasy (Qiagen, Carlsbad, USA). The RT-QPCR was performed with the following forward and reverse primers: GLUT2, 5′-CTCGGGCCTTACGTGTTCTT-3′ and 5′-TAGGCAGCTCATCCTCACACA-3′; CAP, 5′-CGCTGCTCCGACAGGTG-3′ and 5′-CTCGAAGTGCCAAACCAT-3′. The reaction was carried out using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, USA) with CYBER Green I according to the manufacturer's instructions.

Statistical analysis

The data were presented in the mean±SEM format and compared by one-way ANOVA and Tukey's post-hoc comparison. *P* values of less than 0.01 were considered statistically significant. The analysis was conducted using Prism software (Version 4.0; GraphPad Software, San Diego, USA).

Results

Ligand activation of endogenous PPARγ

To test whether endogenous PPARα and γ are activated by their specific ligands, INS-1 cells were transiently transfected with a PPRE-reporter plasmid and treated with various ligands. As shown in **Fig. 1**, BRL 49653, a specific ligand for PPARγ, increased the luciferase activity up to 2.5-fold in a dose-dependent manner, whereas Wy 14643, a specific ligand for PPARα, had no effect on reporter gene activity. Notably, BRL 49653-stimulated luciferase activity was increased 4-fold in the presence of 9-cis-retinoic acid, an endogenous ligand for RXR ($P<0.01$ vs. control). These results not only indicated the presence of PPARγ in INS-1 cells, but also demonstrated that the activation of PPARγ resulted from ligand-dependent heterodimerization of RXR and PPARγ.

Elevated glucose-stimulated insulin secretion (GSIS) by ligand-dependent activation of PPARγ

To investigate the function of PPARγ in the regulation of GSIS, the interaction between GSIS, PPARγ ligands and PPARγ activity was studied. We observed that BRL 49653 enhanced GSIS up to 2-fold compared with the cells incubated with 20 mM glucose only, and overexpression of PPARγ further enhanced GSIS to 2.5-fold (**Fig. 2**). These observations strongly suggested that PPARγ ligand BRL 49653 can elevate GSIS by activating both endogenous and exogenous PPARγ.

The effects of Wy 14643 (a PPARα ligand) and PPARα

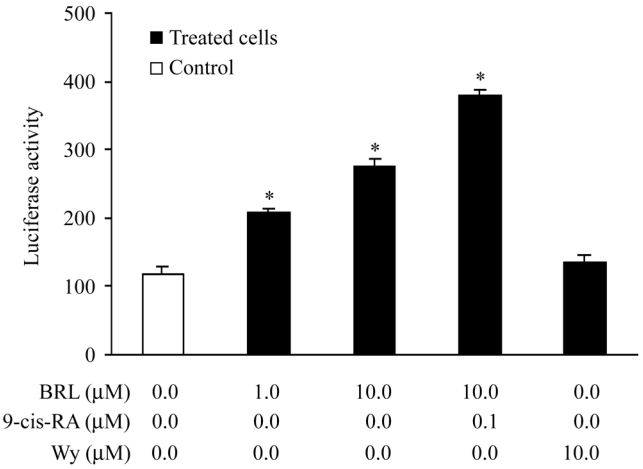

Fig. 1 Ligand activation of endogenous peroxisome proliferator-activated receptors (PPAR)

INS-1 cells were transiently transfected with PPRE3-TK-Luc, a reporter construct containing PPRE, and β-gal plasmid. The PPAR ligands BRL 49653 (BRL) and Wy 14643 (Wy), and 9-cis-retinoic acid (9-cis-RA), an endogenous ligand for RXR, were added to the transfected cells and luciferase reporter activity normalized to β-gal was determined. *$P<0.01$ vs. control.

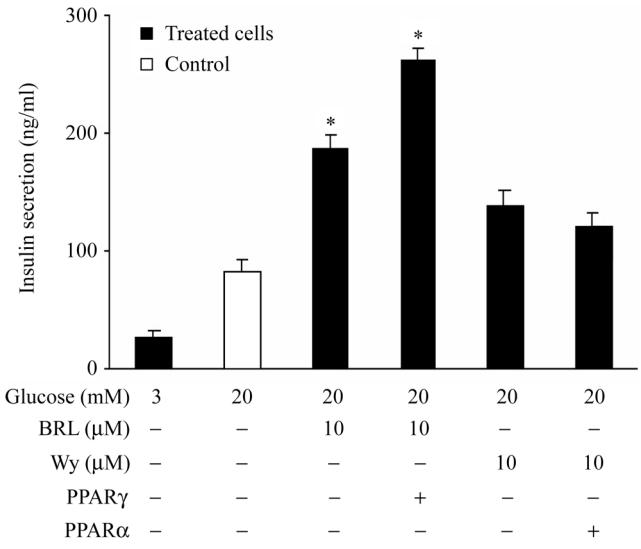

Fig. 2 Ligand-dependent activation of peroxisome proliferator-activated receptors (PPAR) on glucose-stimulated insulin secretion

Cells were transiently transfected with wild-type PPARγ or α plasmid. PPAR ligands BRL 49653 (BRL) and Wy 14643 (Wy) were added to the transfected cells for 24 h and insulin secretion measurement was performed afterwards. *$P<0.01$ vs. control. –, not transfected; +, transfected.

on GSIS were also studied. Compared with BRL 49653, Wy 14643 had less effect on GSIS. Overexpression of PPARα have no effect on GSIS. Combining these results with the observations shown in **Fig. 1**, we concluded that Wy 14643 could not activate endogenous or exogenous PPARα in INS-1 cells.

Function of PPARγ on GSIS

The functional role of PPARγ was further investigated by the overexpression of a constitutively active chimeric form of PPARγ, VP-PPARγ, in INS-1 cells. We found that the overexpression of VP-PPARγ resulted in a 2-fold increase of GSIS in the absence of PPARγ ligand (**Fig. 3**). This increment is comparable to the GSIS elevation when cells were treated with PPARγ ligand.

To confirm that PPARγ is directly involved in the regulation of GSIS, INS-1 cells were transiently transfected with PPARγ-S84A, a PPARγ mutant, which contains a point mutation at the serine phosphorylation site to avoid the activation of PPARγ by MAP kinase. As expected, BRL 49653 did not increase GSIS in cells expression PPARγ-S84A to the same extent as that in cells overexpression wild-type PPARγ (**Fig. 3**). These findings provided direct evidence that PPARγ played a functional role in GSIS regulation.

PPARγ target genes on GSIS

A previous report has identified the promoter region of the GLUT2 gene containing PPRE, which indicates that PPAR may regulate the expression of the GLUT2 gene [24]. To further explore the molecular mechanism of GSIS regulated by PPARγ, we investigated the role of PPARγ in GLUT2 gene activity regulation, the INS-1 cells were transiently transfected and overexpressed with wild-type PPARγ or PPARα plasmid and treated with BRL 49653 or Wy 14643, and then exposed to 3 mM glucose for 2 h, followed by challenged with 20 mM glucose for 2 h as described in materials and methods. We observed that the expression of the GLUT2 gene induced by BRL 49653 was increased up to 5-fold by both endogenous and exogenous PPARγ when the cells were exposed to high glucose concentration (20 mM) for 2 h (**Fig. 4**).

We further examined the expression level of CAP that is known to facilitate GLUT4 translocation in insulin-sensitive tissues [27] with the same research system as above. The CAP expression was found to be induced by BRL 49653 and enhanced by overexpression of PPARγ (**Fig. 4**). These observations suggested that GLUT2 and CAP were involved in the GSIS pathway regulated by PPARγ.

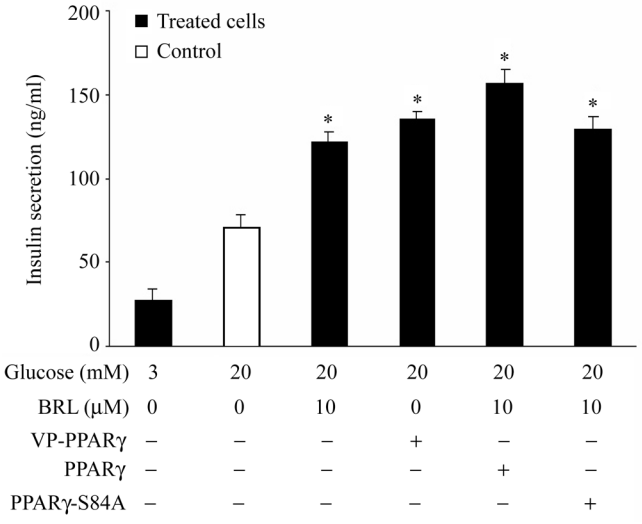

Fig. 3 Peroxisome proliferator-activated receptor (PPAR) gamma function on glucose-stimulated insulin secretion

Various forms of PPARγ were overexpressed in INS-1 cells along with the addition of 10 μM BRL 49653 (BRL) and insulin secretion measurement was performed afterwards. **P*<0.01 vs. control. –, not transfected; +, transfected.

Discussion

There have been many reports on the biological role of PPARα and γ in pancreatic β-cells. PPARs have been reported to express in the human pancreatic islet cells, rodent pancreatic islet cells, INS-1 cells and insulin-producing cell lines including HIT-T15 [28–30]. Moderate amounts of PPARγ are expressed in pancreatic β cells, and its expression is increased in the diabetic state [28, 31]. But the fundamental role of PPARγ in β-cells is not fully understood. Reports on the effects of PPARγ on insulin secretion are contradictory. PPARγ agonists can protect the pancreatic β cells from apoptosis and restore the function of β cells, including GSIS [23]. However, it is reported that PPARγ agonists can also decrease insulin secretion in diabetic animal models [32]. The activation of PPARγ did not improve insulin secretion in isolated human islets [33,34]. Our results suggested that the activation of PPARγ elevated GSIS and increased the expression of the GLUT2 and CAP genes in rat pancreatic β-cell line, INS-1 cells.

Glucose is the most important physiological stimulus for insulin secretion, and the process requires glucose sensing [35]. The glucokinase in pancreatic β-cells (β-GK) is the rate-limiting step in glycolitic flux for insulin secretion, and a small change in β-GK activity sharply affects the threshold for GSIS [36]. GLUT2 is known to play an im-

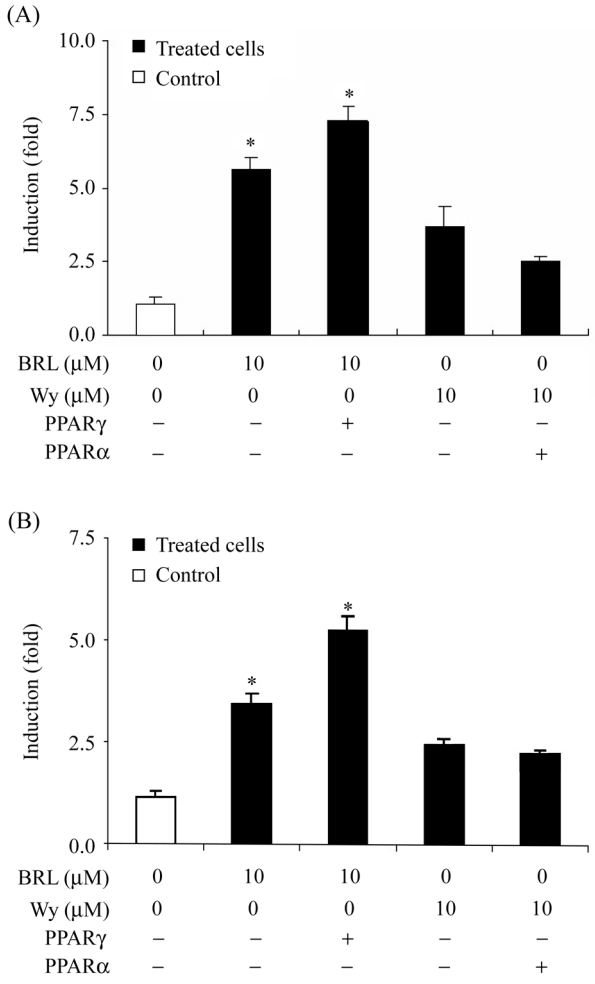

Fig. 4 Effects of peroxisome proliferator-activated receptor (PPAR) gamma-targeted genes on glucose-stimulated insulin secretion

(A) Glucose transporter gene GLUT2 mRNA extracts and (B) Cb1-associated protein (CAP) mRNA extracts from expression plasmids transfected and/or ligands treated-cells were analyzed by real-time quantitative polymerase chain reaction to detect the expression of GLUT2 and CAP genes. **P*<0.01 vs. control. BRL, BRL 49653; Wy, Wy 14643; –, not transfected; +, transfected.

portant role in allowing rapid equilibration of glucose across the plasma membrane. However, it is also essential in GSIS because normal glucose uptake and subsequent metabolic signaling for GSIS can not be achieved without GLUT2. The expression of GLUT2 and β-GK is decreased in diabetes subjects before the loss of GSIS. The β-cell-specific knockout of the GLUT2 or GK gene results in infant death because of severe hyperglycemia [37].

The direct involvement of PPARγ in GSIS was tested by a PPARγ construct which has constitutive activity. Transient transfection of this chimeric receptor has been shown to increase GSIS up to 2-fold in the absence of specific ligands, which suggests that this elevation was directly mediated by PPARγ. In addition, insulin is a stimulator in the MAP kinase signaling pathway. It has been shown that PPARγ activity is downregulated by MAP kinase in preadipocyte 3T3-L1 cells [6]. One possible explanation for such downregulation is that the secreted insulin released into culture media may cause feedback inhibition of PPARγ activity by the phosphorylation of the serine residue at amino acid position 84 in INS-1 cells. However, our mutant PPARγ-S84A construct showed no significant effect on PPARγ activity by the insulin-activated MAP kinase pathway in INS-1 cells. It may provide the information regarding the differential biological effects of PPARγ activity between 3T3-L1 preadipocyte and insulin-producing cells.

GLUT2 is a major form of glucose transporter in pancreatic β-cells and plays a key role in GSIS. Suppression of GLUT2 in pancreatic β-cells is correlated with the loss of high-Km glucose transport and GSIS [38]. Several approaches, including antisense blocking of GLUT2 activity and GLUT2-null islets, have suggested an exclusive role of GLUT2 on glucose uptake, utilization and signaling in pancreatic islets [37,39,40]. Our approach of either stimulating endogenous PPARγ receptor by specific ligands, or overexpressing the constitutively active receptor, suggested that these manipulations induced GLUT2 gene expression, thus demonstrating a direct link with elevated GSIS. This finding is also consistent with an earlier report that the promoter of GLUT2 contains a functional PPRE [24].

Significant progress has been made over the past several years to address the role of insulin receptor on pancreatic β-cells [41]. Interestingly, in mice, the tissue-specific knockout of the insulin receptor in muscle failed to produce diabetes, but the disruption of the gene in β-cells produced a diabetic phenotype [42]. The positive coupling between insulin secretion and insulin receptor action has been suggested by way of PI3 kinase-dependent action [27]. CAP, which associates with Cb-l proto-oncoprotein in a PI3-kinase-independent pathway through insulin receptor signaling, appears to be induced during the adipocyte differentiation by the PPARγ ligands treatment. In the present study, we first reported that CAP was expressed in INS-1 cells. The role of CAP in INS-1 cells is still unknown, and GLUT2 does not undergo insulin-stimulated translocation as compared to GLUT4 in non-insulin producing cells [43,44]. The role that CAP might play in the post-insulin receptor signaling pathway and in promoting GLUT2 translocation in INS-1 cells needs to be investigated.

Our study also revealed distinct mechanisms of glucose-induced insulin secretion, which were mediated by PPARα and PPARγ. Our results suggested that the overexpression of PPARα had no incremental effects on GSIS or the expression of GLUT2 and CAP genes. It is consistent with our findings that the elevated glucose markedly downregulated the expression of the PPARα gene in pancreatic β-cell [45]. The action of glucose on PPARα mRNA expression occurs in less than 2 h and does not require *de novo* protein synthesis. The rapidity of this effect and the absence of a requirement for protein synthesis indicate that the PPARα behaves as an early response gene in INS-1 cells [45].

Rosiglitazone, as well as other TZD antidiabetic drugs, is known to improve insulin resistance by reducing hyperglycemia, hyperinsulinemia and hypertriglyceridemia in human and rodent. Our findings provide a new evidence that PPARγ directly acts on the pancreatic β-cells to enhance GSIS.

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Edited by
Shang-Quan ZHU