Rosiglitazone reverses insulin secretion altered by chronic exposure to free fatty acid via IRS-2-associated phosphatidylinositol 3-kinase pathway[1]

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ABSTRACT

AIM: To study the effect of rosiglitazone (RSG) on insulin secretion in isolated pancreatic islets under chronic exposure to free fatty acid (FFA) and to investigate the potential signaling mechanism of RSG action. **METHODS:** Rat pancreatic islets were cultured with or without FFA (2 mmol/L, oleate:palmitate, 2:1) in the presence or absence of RSG (0.05-10 μmol/L). The insulin release was measured by radioimmuoassay, the expression level of insulin receptor substrate-2 (IRS-2) protein and the association of IRS-2 with p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) were determined by immunoprecipitation and Western blot. **RESULTS:** The islets exposed to high FFA concentration showed an increased basal and a decreased glucose-induced insulin release as compared with control islets ($P<0.01$). IRS-2 protein level was decreased by 65 % ($P<0.01$) and the association of IRS-2 with p85 subunit of PI 3-kinaseand was decreased by 73 % ($P<0.01$). When islets were cultured with FFA in the presence of RSG 5 μmol/L, both basal and glucose-induced insulin secretion were reversed to a pattern of control islets ($P<0.01$, $P<0.05$). The addition of RSG in the cultured medium increased significantly the expression of IRS-2 protein by 2.6 fold ($P<0.01$) and the association of IRS-2 with p85 by 2.7-fold ($P<0.01$) as compared with islets incubated with FFA alone. The effects of RSG on insulin secretion were blocked by a PI 3-kinase inhibitor, wortmannin. **CONCLUSION:** The effects of RSG on insulin secretion could be mediated through an IRS-2-associated PI 3-kinase signaling pathway.

INTRODUCTION

Type 2 diabetes mellitus is characteristic by impaired insulin secretion and diminished peripheral insulin sensitivity. Continuing loss of β-cell function is the underlying cause of deteriorating metabolic control in people with type 2 diabetes. Fatty acids could play a role in the reduction of β-cell insulin secretion[1]. Chronic elevation of free fatty acids (FFA) is known to inhibit insulin secretion and these abnormalities occur frequently in diabetic patients and negatively affect β-cell function[2]. The prevention of lipotoxicity could represent a new therapeutic strategy to preserve insulin se-

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cretion in type 2 diabetic patients. Thiazolidinediones (TZD) have been shown to enhance sensitivity to insulin in the muscle, adipose tissue, and liver by activating a nuclear receptor, peroxisome proliferator-activated receptor (PPAR)-γ, resulting in an improvement of insulin-mediated glucose disposal[3]. Rosiglitazone (RSG) is a new antidiabetic agent of the TZD class. Whether RSG also affects insulin secretion from pancreatic β-cells, is still unknown. Therefore, this study was designed to determine the effect of RSG on insulin secretion in isolated pancreatic islets chronically exposed to FFA and to examine the potential signaling mechanism of RSG action.

MATERIALS AND METHODS

Chemicals RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco-BRL. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad (Richmond, CA). Rabbit polyclonal-anti-IRS-2, and anti-p85 antibodies used for Western blotting were purchased from UBI (Lake Placid, NY). HRPO-anti-rabbit antibody and ECL- reagents were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Collagenase V, oleate and palmitate, wortmannin, UO126, approtinin, pepstatin, and other reagents were from Sigma.

Islets isolation Male Sprague-Dawley rats 200-250 g (Grade II, Certificate No 19-050, from Department of Experimental Animal, Tongji Medical College, Huazhong University of Science and Technology, China) were anesthetized, and pancreatic islets were isolated from the pancreata by the collagenase method[4]. Briefly, after cannulation of the common bile duct and instillation of 10 mL chilled RPMI-1640 medium containing collagenase V 1 g/L, the pancreas was removed and digested at 37 ℃ in a shaking water bath for 30 min, followed by dilution and washing with PRMI-1640 medium containing 0.5 % bovine serum albumin (BSA). Islets from pancreas digest were further purified by centrifugation gradient, and placed into culture dishes. By this technique, 300-400 islets were isolated from each pancreas.

Cells culture The purified islets were first cultured overnight with glucose 2.8 mmol/L in Dulbecco's RPMI-1640 medium containing 10 % FBS, 1 % panicillin/transtomycine at 37 ℃ in a 5 % CO_2-95 % air atmosphere with 85 % relative humidity. Cells then were cultured for 48 h in the presence or absence of long-chain FFA (oleate:palmitate, 2:1) 2 mmol/L in a culture medium containing 2 % bovine serum albumin (BSA). Islets were then cultured for another 24 h in the presence or absence of concentrations of RSG (RSG^+/ RSG^- group). In RSG^+ group, cells were preincubated with wortmannin 100 nmol/L or UO126 100 μmol/L for 30 min prior to the addition of RSG to the medium (Tab 1).

Insulin release At the end of the culture, the islets were washed twice in Krebs-Ringer HEPES buffer (NaCl 115 mmol/L, KCl 5.4 mmol/L, $CaCl_2$ 2.38 mmol/L, $MgSO_4$ 0.8 mmol/L, Na_2HPO_4 1 mmol/L, HEPES 10 mmol/L, 0.5 % BSA, pH 7.4). Groups of 2 purified islets were then incubated with glucose 5.6 or 16.7 mmol/L for 1 h, and then insulin was measured in the medium using radioimmunoassay. Results were expressed as insulin release in the medium ($\mu U \cdot h^{-1}$per islet).

Immunoprecipitation and Western blot For immunoprecipitation and Western blot, after the end of the culture with RSG, cells were immediately homogenized with ice-cold lysis buffer (pH 7.4) 1 mL containing Tris 20 mmol/L, NaCl 137 mmol/L, edetic acid 1 mmol/L, natriumfluorid 1 mmol/L, natriumvanadat 2 mmol/L, 1 % NP-40, phenylmethylsulfonyl fluoride 1 mmol/L and aprotinin 2 mg/L, leupeptin 2 mg/L and pepstatin 2 mg/L. The insoluble material was removed by centrifugation at 12 000×*g*, 4 °C in a Sigma 3 K18 rotor (Sigma) for 60 min, and the supernatant was used as a sample.

Determinations of IRS-2 protein expression level and the interaction of p85 subunit of phosphatidylinositol (PI 3-kinase) with IRS-2 were performed by immunoprecipitation and immunoblotting. Protein samples 500 μg were subjected to immunoprecipitation using specific anti-IRS-2 antibodies conjugate at 4 °C for 2 h. Subsequently, the protein-antibody complexes were precipitated with protein A-Sepharose for another hour. Samples were rotated at 4 °C and then washed three times with lysis buffer. The precipitates were treated with 30 mL laemmli sample buffer and denatured at 95 °C for 10 min. The samples were then subjected to 7.5 % SDS-PAGE and proteins were analyzed.

Immunoprecipitated blots were incubated overnight at 4 °C with rabbit anti-IRS-2 or anti-p85 antibody 1mg/L (1st antibody) in 2.5 % nonfat dried milk. Subsequently, the blots were washed 3 times in 0.05 % Tween-20, Tris-HCl 10 mmol/L, NaCl 150 mmol/L (pH 7.5) for 15 min. The blots were then incubated with 1: 6500 HRPO-conjugate (2nd antibody) for 1 h at room temperature

and washed 3 times again as described above, and then incubated with ECL-Western blotting protocol detection reagent for 1 min at room temperature and immunoreactive bands were visualized. Band intensities were quantitated by Image Quant (Molecular Dynamics).

Statistical analysis The data were expressed as mean±SD. The statistically significant difference between experimental groups was assessed by Student's *t*-test.

RESULTS

Insulin release In control rat islets, basal insulin release (glucose concentration 5.6 mmol/L) was (9.1± 2.1) μU· h^{-1} per islet and increased significantly in response to glucose 16.7 mmol/L [(71±10) μU· h^{-1}per islet]. In islets preexposed to FFA for 48 h, as expected, basal insulin secretion was significantly increased (P<0.01), and glucose-stimulated insulin release was markedly reduced (P<0.01). When these islets were cultured for an additional 24 h in the presence of RSG 5 μmol/L, a clear reversal of the insulin release pattern to control conditions was observed. Basal insulin release was reduced and glucose-stimulated insulin release was increased compared with islets cultured with FFA alone (Tab 1). The dose-effect experiment of RSG showed that the effect of RSG was dose-dependent within 0.05-5 μmol/L physiological concentration and maximal at a concentration of 5 μmol/L (Fig 1). Therefore, the following experiments were performed with RSG 5 μmol/L. In contrast, RSG (0.05-10 μmol/L) did not affect either basal or glucose-induced insulin release in control islets (Fig 1).

Tab 1. Effect of RSG on insulin secretion. ***n*****=4. Mean±SD.** ^{b}P**<0.05,** ^{c}P**<0.01** ***vs*** **control.** ^{e}P**<0.05,** ^{f}P**<0.01** ***vs*** **FFA group.** ^{h}P**<0.05** ***vs*** **FFA+RSG group.**

Groups	Basal insulin release content/mU· h^{-1} per islet	Glucose-stimulated insulin release content/mU· h^{-1} per islet
Control	9.1±2.1	71±10
Control+RSG	8.6±2.1	75±11
FFA	25±4^{c}	44±7^{c}
FFA+RSG	13±3^{f}	63± 8^{e}
FFA+RSG+Wort	17.1±2.3^{be}	46±10^{bh}
FFA+RSG+UO	12.5±2.2^{f}	64±8^{f}

RSG : rosiglitazone 5 μmol/L; FFA: free fatty acid 2 mmol/L; Wort: wortmannin 100 nmol/L; UO: UO126 100 μmol/L.

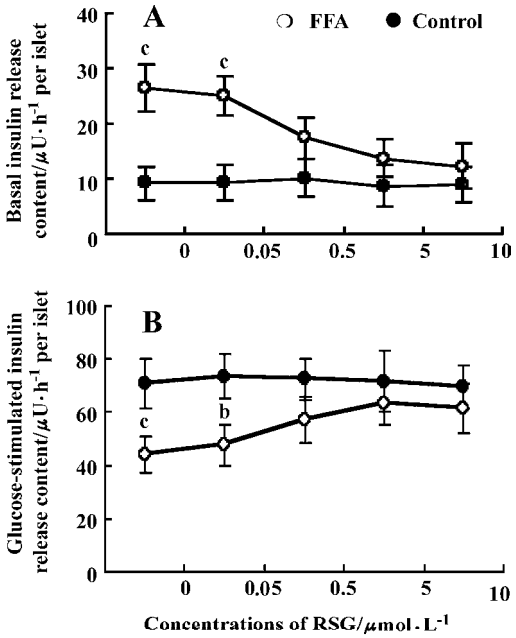

Fig 1. The dose-effect experiments of RSG. ***n*** **=4. Mean± SD. A: Basal (glucose 5.6 mmol/L) insulin release; B: Glucose-stimulated insulin release (glucose 16.7 mmol/L).** ^{b}P**<0.05,** ^{c}P**<0.01** ***vs*** **control .**

Role of signaling transduction inhibitors in RSG effect on insulin secretion To determine the possible signaling pathways leading to the effect of RSG on insulin secretion, we examined the effect of RSG on insulin secretion in the presence of intracellular signaling transduction inhibitors. The inhibitors used were wortmannin, which inhibits PI 3-kinase, and UD126, a MAPK inhibitor, which binds to MEK1 and MEK2 and inhibits both molecules. Wortmannin 100 nmol/L blocked significantly the glucose-stimulated insulin releases improved by RSG (P<0.05, Tab 1), and also blocked partly basal insulin release in the presence of RSG (P=0.0704), indicating a possible role of PI 3-kinase pathway in the effect of RSG. In contrast, MAPK inhibitor UO126 had not any influence on insulin secretion improved by RSG.

Expression level of IRS-2 protein and the association of IRS-2 with p85 subunit of PI 3-kinase To confirm further whether the effect of RSG on insulin secretion is mediated via IRS-2–associated PI 3-kinase pathway, the expression level of IRS-2 protein and the association of IRS-2 with p85 subunit of PI 3-kinase were determined. The blots with immunopresipitates for IRS-2 were immunoblotted with anti-IRS-2 or anti-p85 antibodies. As expected, after expo-

sure of islets to FFA 2 mmol/L for 48 h, the expression level of IRS-2 protein was decreased by 65 % ($P<0.01$), the association of IRS-2 with p85 subunit of PI 3-kinase was correspondingly decreased by 73 % ($P<0.01$). The addition of RSG 5 μmol/L reversed significantly the decrease of expression level of IRS-2 protein and the association of IRS-2 with p85 subunit of PI 3-kinase induced by chronic exposure to FFA. The expression level of IRS-2 protein was increased by 2.6-fold ($P<0.01$), and the association of IRS-2 with p85 subunit of PI 3-kinase was increased 2.7-fold ($P<0.01$, Fig 2). RSG alone did not affect the association of IRS-2 with p85, the expression level of IRS-2 protein was slightly increased but not significantly.

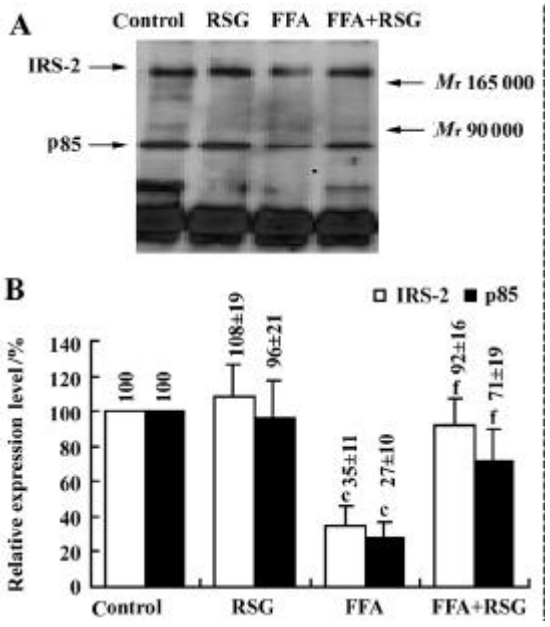

Fig 2. The expression of IRS-2 protein and the association of IRS-2 with p85 subunit of PI 3-kinase. A typical blot for scanning densitometry by Image Quant (Molecular Dynamics) is shown above (A). n=4. Mean±SD. They are expressed as relative to control values, which were set at 100 % (B). $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* FFA group. RSG: rosiglitazone 5 mmol/L; FFA: free fatty acid 2 mmol/L.

DISCUSSION

Continuing loss of β-cell function is the underlying cause of deteriorating metabolic control in people with type 2 diabetes. Fatty acids could play a role in the reduction of β-cell insulin secretion. The present study observed the effect of elevated FFA on insulin secretion in isolated rat pancreatic islets by chronic exposure to elevated FFA. The results showed that, both basal and glucose-stimulated insulin release were impaired, confirming a lipotoxicity on β-cells.

The failure of β-cell function is believed to be attributable to the apoptosis of β-cells in response to increased FFA or to an accumulation of triglycerides in the β-cells[1,2]. The FFA excess in β-cells increases the *novo* ceramide synthesis[5] and nitric oxide formation[6], while reducing the antiapoptotic protection of β-cells[7]. In the present study, we found that the chronic exposure of islets β-cells to elevated FFA induced a significant decrease in the expression of IRS-2 protein and the association of IRS-2 with PI 3-kinase. This suggests that the decrease in the β-cell insulin secretion induced by chronic exposure to FFA could be related to the inhibition of IRS-2-associated PI 3-kinase activation.

The present study tested the hypothesis that RSG could modify β-cell secretory abnormalities induced in isolated rat pancreatic islets by chronic exposure to elevated FFA levels. Our results showed that, when RSG was added to the culture medium, insulin secretory abnormalities were restored, both basal and glucose-induced insulin release were restored to the pattern observed in control ielets. This protective effect of RSG on islet β-cells implies that part of the therapeutic action of TZD in human type 2 diabetes could be the result of the prevention of β-cell function loss and the restoration of insulin secretory capacity in addition to its well-known effects on the peripheral insulin target tissues.

The TZD compound troglitazone has also been reported to improve β-cell function in the fat-laden islets of Zucker diabetic fatty (ZDF)[8], which relate to prevention of intracellular lipid accumulation, protection of beta cells from lipoapoptosis, enhancement of fatty acid oxidation, decrease in nitric oxide formation, or protection of islets from cytokine toxicity. However, in these studies, the effects of TZD on insulin secretion are explained most through intracellular metabolic alteration. The signaling mechanism of TZD effect on insulin secretion from β-cells is still unclear.

To investigate further the possible signaling mechanism of RSG action, we observed the expression of IRS-2 and the association of IRS-2 with p85, and tested also the effect of wortmannin, a PI 3-kinase inhibitor. These results indicated that the improved insulin secretion function by RSG was correlated with an increase in the expression level of IRS-2 protein and the IRS-2-associated PI 3-kinase activation, suggesting that the effect of RSG on insulin secretion could partly via the

PI 3-kinase pathway. At least, the potentiation effect of RSG on glucose-stimulated insulin secretion may be mediated by an IRS-2- associated PI 3-kinase pathway.

IRS proteins are the major members mediating intracellular signaling transduction, which become docking sites for Src homology 2-containing enzymes like PI 3-kinase after tyrosine phosphorylation. IRS-2 plays an important role in the mediation of β-cells insulin secretion[9,10]. Indeed, in the homozygous IRS-2-deficient mice (IRS-2-/- mice), insulin secretion is significantly decreased[11]. PI 3-kinase as a critical component in the intracellular signaling transduction, mediates the regulation of a broad array of biological responses by various hormones[12]. Rosiglitazone had been reported to increase IRS-1 and IRS-2 levels and insulin effects on IRS-1- and IRS-2-dependent PI 3-kinase, thereby enhancing glucose transport in 3T3/L1 adipocytes[13]. Interestingly, a recent study also showed that the direct stimulation of RSG on glucose-induced insulin secretion might be mediated via PI3-kinase pathway, because the potential effect of RSG on insulin secretion is blocked by an another PI 3-kinase inhibitor, LY294002[14]. Indeed, several recent studies have indicated that β-cells express components of insulin signaling systems as IRS-1, IRS-2, and PI 3-kinase[15]. Insulin-induced insulin secretion also involved insulin receptors, IRS-1, and PI 3-kinase activation [15]. These investigations suggest that insulin signaling transduction PI 3-kinase pathway mediates not only insulin metabolic action in peripheral tissues, but also may play an important role in insulin secretion of β-cells. Therefore, based on these foregoing facts, together with the present results, it indicates that the IRS-2 associated PI 3-kinase pathway may be involved in restored effect of RSG on insulin secretion altered by chronic exposure to FFA.

PPAR-proteins, the members of the nuclear receptor family, are expressed in the rat islet and in a β-cell line, MIN6. Shimabukuro[16] reported that the stimulation of PPAR by RSG induced mRNA expression of the p85 subunit of the PI 3-kinas and of the uncoupling protein-2 genes in the pancreatic islets of Zucker diabetic fatty rats. However, it is unclear whether the activation of these proteins also mediates the effect of RSG on the expression of IRS-2 protein and PI-3-kinase activation. We did not find a direct effect of RSG on insulin secretion, because RSG did not affect insulin secretion in the control islets, suggesting that the effects of RSG on insulin secretion were secondarily attributed to the amelioration of lipotoxicity.

In conclusion, our data indicate that RSG may reverse the insulin-secretory capacity of β-cells altered by chronic exposure to free fatty acid, supporting the hypothesis that RSG protects islet β-cells from lipoapoptosis. This prevention of lipotoxicity may represent a new therapeutic strategy to preserve insulin secretion in type 2 diabetic patients. The effect of RSG on insulin secretion may be mediated through an IRS-2-associated PI 3-kinase signaling pathway.

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