Effects of Metformin on Rosiglitazone-Induced Cardiac Hypertrophy in Mice

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Thiazolidinediones (TZD) can cause adipose tissue accumulation and myocardial hypertrophy. This study aimed to determine if combined Metformin (Glucophage) and Rosiglitazone (Avandia) could reduce the risk of heart failure caused by Rosiglitazone in BALB/c mice. BALB/c mice were treated with oral Rosiglitazone/Metformin twice daily for four weeks. Metformin or Rosiglitazone alone and non-treated mice acted as double control. Myocardial hypertrophy and associated side effects of the combined therapy were determined through isolated heart and body weights. Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were applied to evaluate expression of sulfonylurea receptor 2A (SUR2A) and Kir 6.2. The activities of peroxisome proliferator activated receptor α (PPARα) in the myocardium were also observed. Rosiglitazone/Metformin decreased body weight gain and food intake, and inhibited an increasing adipose ratio but did not reduce myocardial hypertrophy. Rosiglitazone increased Kir6.2/SUR2A, Kir6.2/SUR2B, and PPARα gene expression. The Rosiglitazone/Metformin combination further increased these gene expressions, especially PPARα. Metformin inhibits obesity but has no effect in reducing myocardial hypertrophy caused by Rosiglitazone. Whether Metformin can reduce side effects of TZDs in humans warrants further study.

Key words Metformin; Rosiglitazone; myocardial hypertrophy; obesity; adipose accumulation

The underlying pathogenesis of type 2 diabetes (T2DM) is very complex. The choice of oral hypoglycemic agents to use for treating T2DM is based on the patient’s clinical characteristics and symptoms. Metformin, a biguanide, can improve the biological response of insulin and reduce insulin resistance. It inhibits hepatic glucose synthesis and lowers triglyceride without promoting body weight gain. Thus, it is widely used for treating T2DM-associated obesity.1,2) Rosiglitazone, a thiazolidinedione (TZD), improves insulin sensitivity in adipose tissue and muscle. It has a secondary effect on liver glucose metabolism.3) However, an important adverse effect is TZD-induced cardiac hypertrophy.4,5) It is also associated with increased risk of heart failure in the clinical setting.6) The combined regimen of TZD and Metformin for glycemic control has been shown to have clinical benefit and efficacy. It remains unknown if this regimen can reduce TZD-induced cardiac hypertrophy.7,8)

Diabetic Zucker (fa/fa) or glucose-fed rats have symptoms similar to T2DM patients with high serum glucose and insulin levels, insulin resistance of peripheral tissues, and hypertension. Therefore, diabetic Zucker rats have been treated as an animal model for T2DM and hypertension.9) In a previous study, T2DM rats were treated with combined Rosiglitazone (Avandia) and Metformin (Glucophage) twice a day for 4 weeks to assess the effect on induced insulin resistance. However, myocardial hypertrophy was rare in these rats with the short-term treatment of the preliminary study. It can be posited that beneficial effects of TZD on metabolism may overcome the cardiac effects or genetic alterations in this combination therapy.

Thus, the objective of this study was to investigate whether Metformin in a combination regimen could reduce rosiglitazone-induced cardiac hypertrophy in BALB/c mice by determining the proportions of adipose tissue and isolated heart weight, as well as gene expressions of Kir6.2/sulfonylurea receptor 2A (SUR2A), Kir6.2/SUR2B, and peroxisome proliferator activated receptor α (PPARα).

MATERIALS AND METHODS

Experimental Animals Male BALB/c mice (8 weeks old) were maintained at the National Cheng Kung University Lab Animal Center, in a temperature- and humidity-controlled (25±1°C and 60±5%) environment with strict 12-h light–dark cycle and free access to food and water. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act.

Experimental Design After the acclimatizing period, the mice were divided into four treatment groups: TZD group, 10 mg/kg Rosiglitazone (Avandia); TZD+Metformin (MET) group, 10 mg/kg Rosiglitazone and 100 mg/kg Metformin (Glucophage); MET group, 100 mg/kg Metformin; and the control group, normal mice. All drugs were given by gastric tube feeding twice daily for 4 weeks. Daily weight and food intake were recorded and compared with the control group weekly during the experimental periods. The mice were sacrificed after 4 weeks through an intra-peritoneal lethal dose injection of 150 mg/kg pentobarbital. The weight of isolated heart and adipose tissue were measured, and gene expressions of Kir6.2/SUR2A, Kir6.2/SUR2B, and PPARα in isolated heart were also examined.

Ingestion Behavior After 6 h of starvation, each mouse was given regular fodder (approximately 5.0 g) in each group. The total consumptions per hour for 3 h were

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recorded.

**Western Blot** Isolated heart tissues were homogenized with Waring Blender, and the expression of SUR2A, Kir6.2, and PPARα were analyzed by Western blot. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separated the proteins, and Western blot analysis was performed using indicated antibodies. The blots were incubated with appropriate peroxidase-conjugated secondary antibodies. After removing secondary antibodies, the blots were washed and developed using the enhanced chemiluminescence (ECL)–Western blotting system. Densities of the obtained immunoblots were quantified using ImageQuant. The blots were probed with a mouse monoclonal actin antibody to ensure a constant amount of protein loaded into each lane of the gel.

**Reverse Transcription Combined with Polymerase Chain Reaction (RT-PCR)** The mRNA expression of myocardial SUR2A, Kir6.2, and PPARα were analyzed by RT-PCR. Overall, RNA in tissue was isolated by Ultraspec™-II RNA extraction system and concentration was determined by absorbance at 260 nm. Total RNA (2 μg) extracted from skeletal muscle was then reverse-transcribed in the presence of an anchored oligo-p(dT15)-primer at 65°C for dry heat bathing 15 min, and cooling for 10 min. After adding reaction buffer (10X), MgCl₂ (25 mmol/l), deoxyribonucleotide triphosphate (dNTP) (10 mmol/l), RNase inhibitor (40 U/μl), and Avian Myeloblastosis Virus reverse transcriptase and incubation for 2 h at 42°C, millions of copies of the sequence of interest were generated to maximize cDNA. The original RNA template was degraded by GeneAmp PCR system 2400, leaving pure cDNA (Perkin Elmer Inc., U.S.A.). The DNA template contained the region of the cDNA fragment from tissue. Reactive buffer (10X), MgCl₂ (25 mmol/l), dNTP (10 mmol/l), and 5’ and 3’ primers of PPARα were added and then incorporated with Tag DNA polymerase (2.5 units) purified by GeneAmp PCR system 2400. The PCR consisted of a series of 35 cycles in polymerase chain reaction, carried out in three steps: (1) denaturing at 95°C, 30 s; (2) annealing at 50°C, 30 s; and (3) elongation at 72°C, 30 s. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel, and the DNA was detected by UV-trans-illumination after staining with ethidium bromide (0.5 mg/ml) based on 100 bp DNA marker (Promega) to quantify relative strength.

**Data Analysis** Statistical analysis was performed using ANOVA analysis and Newman–Keuls *post-hoc* analysis. Statistical significance was set at *p* < 0.05. Results were expressed as mean± standard error (S.E.).

**RESULTS**

**Body Weight Gain of Mice** The body weight of the control mice increased along with age. After TZD administration for 4 weeks, body weight gain of the study mice was higher than that of the control group (*p* < 0.05). Body weight gain was inhibited by Metformin administration in TZD+MET mice when compared with control group at week 4 (*p* < 0.05). Weight gain was not observed in MET group of mice (Fig. 1).

**Daily Ingestion Quantity** Daily ingestion quantity was recorded to calculate variations in weekly average. The daily ingestion quantity of TZD mice was not different from that of the control groups except at week 4 (*p* < 0.05). The daily ingestion quantity of TZD+MET mice was lower than that of control mice at week 1, week 3, and week 4 (*p* < 0.05) (Fig. 2). We also observed that the daily ingestion quantity of TZD+MET mice was lower than that of TZD treated mice at week 1 and week 3 (*p* < 0.05) (Fig. 2).

**Ingestion Behavior** Ingestion quantity within 3 h in TZD mice was significantly lower than that of controls in the 2nd (*p* < 0.05), 3rd (*p* < 0.05) and 4th (*p* < 0.05) weeks (Fig. 3). The decrease in ingestion behavior was decreased slightly by Metformin administration (Fig. 3).

**Drugs** Drugs were given orally twice daily. TZD, Rosiglitazone (10 mg/kg); TZD+MET, Rosiglitazone (10 mg/kg) and Metformin (100 mg/kg); MET, Metformin (100 mg/kg).

**Fig. 1. Body Weight Variations from Week 1 to Week 4 in Mice** Drugs were given orally twice daily. TZD, Rosiglitazone (10 mg/kg); TZD+MET, Rosiglitazone (10 mg/kg) and Metformin (100 mg/kg); MET, Metformin (100 mg/kg). Results were presented as mean±S.E.M. (*n* = 6). *p* < 0.05 compared to controls. § *p* < 0.05 compared to Week 0. *p* < 0.05 compared to TZD.

**Fig. 2. Weekly Variations of Ingestion Quantity in Mice** Drugs were given orally twice daily. TZD, Rosiglitazone (10 mg/kg); TZD+MET, Rosiglitazone (10 mg/kg) and Metformin (100 mg/kg); MET, Metformin (100 mg/kg). Results were presented as mean±S.E.M. (*n* = 6). *p* < 0.05 compared to TZD. *p* < 0.05 compared to Week 0. *p* < 0.05 compared to TZD.

**Fig. 3. Ingestion Quantities within 3 h from Week 1 to Week 4** Drugs were given orally twice daily. TZD, Rosiglitazone (10 mg/kg); TZD+MET, Rosiglitazone (10 mg/kg) and Metformin (100 mg/kg); MET, Metformin (100 mg/kg). Results were presented as mean±S.E.M. (*n* = 6). *p* < 0.05 compared to controls.
Adiposity Ratio and Isolated Heart Weight

The experimental mice were sacrificed at the end of week 4 to measure the weights of the isolated heart, brown adipose tissue (BAT) at the back, and white adipose tissue (WAT) of the epididymis. The results showed that the proportions of adipose tissue to body weight in TZD mice were higher than in the control group, regardless of brown adipose tissue (1.35±0.11% vs. 1.27±0.04%) or white adipose tissue (1.40±0.12% vs. 1.22±0.07%). The adiposity ratio in TZD+MET mice was significantly lower than in TZD mice (BAT 1.24±0.04% and WAT 1.05±0.08%; p<0.05). The effect of Metformin in adipose reduction was more significant (BAT 0.56±0.06%; WAT 0.67±0.14%) when compared with that of control mice (p<0.05) (Fig. 4). The ratio of heart weight to body weight was not significantly different among the four groups (Fig. 5).

Gene Expression of Kir6.2/SUR2A, Kir6.2/SUR2B, and PPARα in the Isolated Heart

Analysis of mRNA to assess gene expression of subunits (SUR2A and Kir6.2) of potassium channel and peroxisome proliferators-activated receptors (PPARα) in isolated heart was performed by RT-PCR (Fig. 6A). Compared to normal mice, mRNA expression of PPARα increased in TZD mice, which was also noted in TZD+MET and MET mice. However, there was decreased mRNA expression of Kir6.2 in TZD mice. TZD with Metformin increased the mRNA expression of Kir6.2 inversely. The mRNA expression of SUR2A in mice was slightly increased by TZD but raised significantly by TZD+MET (Fig. 6B). Western blotting evaluated cardiac protein expression (Fig. 6B). The PPARα expression of TZD mice was higher than that of control, and was even higher in TZD+MET mice. Metformin had no effect on PPARα protein expression. The protein expressions of SUR2A and Kir6.2 were increased by TZD as compared to the control group. This was also higher in the TZD+MET mice. However, Metformin did not affect potassium channel protein expressions in the heart.

DISCUSSION

In the present study, rosiglitazone (TZD) increases the proportions of adipose tissue (BAT and WAT), which results in cardiac hypertrophy in BALB/c mice. Metformin inhibits the increase of adipose tissue in TZD-MET mice, but does not influence the process of cardiac hypertrophy. PPARα, SUR2A, and Kir6.2 are up-regulated in rosiglitazone (TZD) administered mice, and these up-regulations are even stronger when TZD is given together with Metformin.

Previous reports indicate that TZD has no effect on the treatment of metabolic syndrome but it increases body weight when administered to mice with high-fat diet. On the
other hand, non-TZD selective PPARγ modulator (nTZDps) can effectively reduce the body weight in mice given high-fat diet.\textsuperscript{10} In this study, TZD increases body weight and the proportions of adipose tissue in mice, while TZD combined with Metformin inhibits this body weight gain. Actually, Metformin is known to lower body weight in human subjects.\textsuperscript{1}\n\nIn the present study, weight gain was not observed in Metformin treated mice (Fig. 1) and daily food intake was not raised by Metformin (Fig. 2). Thus, Metformin alone did not decrease the body weight markedly. This difference probably can be considered as the variation of rodent and human. But it needs more studies to clarify in the future.

TZD is known to inhibit the growth of cardiomyocytes in vitro. Paradoxically, it can also cause myocardial hypertrophy, which is observed after PPARγ-activation in TZD administered mice.\textsuperscript{10,11} In this study, heart weight over body weight in normal mice is 0.46±0.03\%, which increases to 0.53±0.04\% when TZD is given. Thus, TZD can cause cardiac hypertrophy. The effect of TZD on cardiac hypertrophy is not affected by Metformin since heart weight over body weight is 0.56±0.04\% in TZD+MET mice. There are no significant differences in this proportion between MET mice and control mice (Fig. 5).

The results suggest that Rosiglitazone combined with Metformin decreases adipose tissue but has no effect on cardiac hypertrophy. It provides an alternative consideration for the clinical application of combined oral diabetes medicine for reducing side effects. There is an association between insulin resistance and abnormal blood lipids, obesity, and type 2 diabetes,\textsuperscript{6,7} but whether or not insulin resistance has a direct effect on cardio-vascular disease is indefinite. The causal relationship between insulin resistance and cardio-vascular disease remains controversial. Type 2 diabetes is considered a risk factor of cardio-vascular disease through increased peripheral vessel resistance and aggravated myocardial burden.\textsuperscript{7,8} Therefore, understanding the relationship between type 2 diabetes and cardio-vascular disease will be helpful in investigating the development of insulin resistance or metabolic syndrome.

Insulin resistance usually involves abnormal lipid and glucose metabolism, and cardio-vascular disease. Lipid metabolism correlates with peroxisome proliferator activated receptors (PPARs), including three subtypes (PPARα, PPARδ, and PPARγ).\textsuperscript{10} PPARs in nucleus binds with 9\textit{-cis} retinoic acid receptor (RXR) to form heterodimers and conjugates peroxisome proliferator responsive element (PPRE) of target genes to activate protein expression related to lipid metabolism. PPARα is distributed mainly in cells with high beta-oxidation rate, such as the liver, kidneys, heart, and muscle, while PPARγ is mainly in fatty cells and PPARδ is in the whole body.\textsuperscript{10,13} When a specific ligand, \textit{i.e.} free fatty acid, fibrates, or thiazolidinedione (TZD) irritates PPARs, lipoprotein lipase is activated to accelerate the absorption of fatty acid and lipid synthesis, and to decrease lipid metabolism, consequently preventing cardio-vascular disease.\textsuperscript{14,15} Therefore, PPARs gene expression contributes to the causal relationship between insulin resistance and cardio-vascular disease.

Insulin resistance is a common pathologic characteristic of diabetes and cardio-vascular disease. If the modulation of PPARs and adenosine triphosphate-sensitive potassium channel (K_{ATP} channel) is investigated, the understanding of diabetes-associated cardiac hypertrophy and obesity will improve the treatment of insulin resistance. Troglitazone (Rezulin) is the earliest insulin sensitizer but it was banned because of serious liver toxicity. The new products, Rosiglitazone (Avandia) and Pioglitazone (Actos), have no cases of liver toxicity but have increased risk of heart failure.\textsuperscript{16—18} It is therefore important to reduce the cardiac side effects of TZD when it is applied clinically. Potassium channels are found in most cells and their malfunction can lead to various diseases, including epilepsy, diabetes, and hypertension. The maintenance of ion channel function has also been investigated. K_{ATP} channel plays an important role in regulating vessel tension and gland secretion. K_{ATP} is composed of an inwardly rectifying potassium channel (Kir) and a sulfonylurea receptor (SUR).\textsuperscript{19} In pancreatic \textit{β} cells, high level of glucose leads to increased ATP production, which in turn binds to the SUR of K_{ATP} channel and results in channel closure and depolarization. The reduced membrane potential, in turn, opens the voltage-dependent calcium channel and increases the intracellular calcium concentration to trigger the exocytosis of insulin.\textsuperscript{20} In the heart, acute myocardial ischemia opens K_{ATP} channels and decreases the flow of calcium into the smooth muscles to dilate vessels and reduce heart contraction. Thus, blood flow to downstream cells of heart tissues will continue, robustly protected from an ischemic insult when the blood supply is cut off entirely and permanently; this is ischemic pre-conditioning. Hyperglycemia or hyper-insulinemia may be the inhibitor of such protection.\textsuperscript{19,20} As a result, K_{ATP} channels may be associated with cardio-vascular disease, and sulphonylureas (\textit{i.e.} glibenclamide and tolbutamide) for long-term treatment of type 2 diabetes can increase the risk of cardio-vascular disease due to their effects on K_{ATP} channels in the heart. In the present study, TZD reduces the expression of K_{ATP} channels, which are even upregulated when TZD is combined with Metformin. Whether Metformin combined with TZD can prevent ischemic injury warrants further study. Otherwise, failure of Metformin to improve the myocardial hypertrophy induced by TZD in mice may be due to several reasons; one is the normal animal but not the diabetic animal was used in this study. However, the real mechanism(s) shall be investigated in the future.

In conclusion, the obtained results suggested a new finding that combination of Metformin with Rosiglitazone decreased body weight gain and/or food intake in addition to a reduction of increased adipose ratio but did not reduce the myocardial hypertrophy in mice. This is helpful for understanding the combined treatments of two agents in clinic.

REFERENCES