Releasing Plasma Volume Blunts Thiazolidinedione-Induced Cardiac Hypertrophy

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Abstract

Aims: Concern of thiazolidinediones (TZDs), including rosiglitazone (Rosi) and pioglitazone, on cardiovascular side effects is increasing in recent years. In animals, TZDs induce cardiac hypertrophy, which has been attributed to the increase in plasma volume or the change of nutrient preference. Thus, we sought to define the causative role of plasma volume expansion in TZD-induced cardiac hypertrophy.

Methods and Results: Treatment by Rosi significantly induced plasma volume expansion and cardiac hypertrophy in wild-type mice, accompanied by reprogramming of hypertrophic genes, cardiomyocyte apoptosis, and Erk1/2 activation. Co-treatment with the diuretic furosemide (Furo) attenuated Rosi-induced cardiac hypertrophy, hypertrophic gene reprogramming, apoptosis, and Erk1/2 activation. In contrast, glucose and lipid metabolism genes in the heart were altered by Rosi, but not reversed by Furo co-treatment, suggesting that the action of TZD on change of nutrient preference in the heart is not the cause of TZD-induced cardiac hypertrophy. Finally, lack of intact ligand binding of Pparg^{P465L/+} mice, but not PPARγ heterozygous knockout of Pparg^{+/−} mice, attenuated both Rosi-induced plasma volume expansion and cardiac hypertrophy, confirming the dependence of Rosi-induced cardiac hypertrophy on plasma volume expansion.

Conclusion: Despite the direct effect of PPARγ activation on metabolic genes in the heart, release of plasma volume was necessary to ameliorate Rosi-induced cardiac hypertrophy and changes in hypertrophic genes and related signals. Our data establish a causal relationship between plasma volume expansion and TZD-induced cardiac hypertrophy.
Introduction

Thiazolidinediones (TZDs), including rosiglitazone (Rosi) and pioglitazone (Pio), are PPAR\(\gamma\) agonists clinically used to treat hyperglycemia associated with diabetes mellitus. However, concern of TZDs on cardiovascular side effects is increasing in recent years. In a large meta-analysis, Nissen and Wolski reported that treatment of Rosi was associated with increased risks of myocardial infarction and cardiovascular death.\(^1\) However, subsequent reports provided variable evidence of the adverse cardiovascular effects of Rosi and Pio.\(^2,\)\(^3\) Although the controversy over Rosi has undermined confidence in developing drugs targeting PPAR\(\gamma\), better understating of the side effects is required to develop safe anti-diabetic therapies targeting PPAR\(\gamma\).

The effects of TZDs on and the role of PPAR\(\gamma\) in heart function and disease have been controversial in rodent and cell models. For example, TZDs inhibit cardiac hypertrophy induced by pressure overload or angiotensin II stimulation in rodent models,\(^4,\)\(^5\) and inhibit cardiomyocyte hypertrophy induced by angiotensin II, endothelin-1, or mechanical stress in cell models.\(^5-7\) Consistent with the inhibitory role of PPAR\(\gamma\) agonists in cardiac hypertrophy, cardiac-specific PPAR\(\gamma\) deletion caused cardiac hypertrophy,\(^8-10\) and PPAR\(\gamma\) heterozygous knockout increased susceptibility to pressure overload-induced cardiac hypertrophy.\(^5\) However, in contrast, several
studies reported cardiac hypertrophy following relatively short-term treatment with Rosi or Pio. Supporting the effect of PPARγ on stimulation of cardiac hypertrophy, cardiac-specific PPARγ overexpression leads to cardiac hypertrophy. Thus, these results suggest that PPARγ agonists have different roles in the development of various types of cardiac remodeling.

Cardiac remodeling and hypertrophy is highly associated with fetal gene reprogramming, including re-expression of natriuretic peptides and switches in contractile proteins and metabolic enzymes. Decreases in fatty acid oxidation and increases in glucose utilization lead to the preference of nutrient usage from fatty acids to carbohydrates in the heart. Interference of fatty acid or glucose utilization in rodents has been shown to induce cardiac hypertrophy or severe heart diseases, suggesting that alteration in energy substrate is sufficient for induction of cardiac hypertrophy. PPARγ activation is known to induce genes involved in lipid uptake and storage, glucose utilization, and energy expenditure in adipose tissue. Moreover, PPARγ activation exhibits a variety of systemic effects, and most remarkably, it directs a greater portion of lipid to adipose tissue. While cardiomyocyte overexpression of PPARγ increases expression of genes involved in fatty acid oxidation and lipid uptake, Rosi-treated mice show attenuation of these genes in the heart. Because PPARγ downstream genes are critical in regulation of glucose and lipid metabolism in the heart, it is reasonable to speculate that PPARγ activation induces
cardiac hypertrophy through the modulation of nutrient metabolism.

In addition to adipose tissue, other tissues, including heart and kidney, express PPARγ at relatively low levels. Thus, TZDs may induce cardiac hypertrophy through either direct actions on the heart, or indirect targets in non-cardiac tissues. This is supported by the study from Duan et al., demonstrating that Rosi continues to cause cardiac hypertrophy in mice with cardiac-specific PPARγ deletion. Thus TZD induces cardiac hypertrophy likely through the action of PPARγ in non-cardiac tissues. Moreover, renal PPARγ activation has been shown to up-regulate ENaCγ, a gene encoding the subunit of sodium transporter. Up-regulation of ENaCγ increases sodium re-absorption and further induces plasma volume expansion. Increased plasma volume has been well documented to induce cardiac hypertrophy and heart failure. Thus, it cannot be excluded that PPARγ activation induces cardiac hypertrophy through the up-regulation of renal ENaCγ and plasma volume expansion.

Although TZD-induced cardiac hypertrophy has been associated with the increase in plasma volume and the change in nutrient preference, the causative roles have not yet been established. Thus, we aim to address whether TZD-induced cardiac hypertrophy is directly mediated through plasma volume expansion. In the current study, we released TZD-induced volume overload by
feeding mice the diuretic furosemide (Furo), and examined PPARγ dependence on both PPARγ heterozygous knockout (\(Ppar^+/\)) and ligand-binding defective PPARγ mutant (\(Ppar^{P465L/+}\)) mice.\(^{20}\) Finally, we also tested the “on-target” and “off-target” effects of volume expansion in TZD-induced cardiac hypertrophy.
Methods

Mice

Pparg<sup>P465L/+</sup> mice<sup>20</sup> with the dominant negative P465L mutation in the ligand-binding domain of PPARγ were maintained on a C57BL/6 genetic background. Pparg<sup>+/−</sup> mice on a 129S6 background (kindly provided by Dr. Ronald Evans at the Salk Institute) were mated with wild-type C57BL/6 mice to obtain F1 littermates for the study. Because TZD is predominantly used for the treatment of type 2 diabetes, the mouse model with high-fat diet-induced diabetes was chosen. All mice at 8 weeks of age were fed with a high-fat diet (58R2; TestDiet, Richmond, IN, USA) or a high-fat diet blended with Rosi (10 mg/kg/day) or Pio (40 mg/kg/day) for 4 weeks. Furo (0.1 mg/ml, Sigma-Aldrich, St Louis, MO, USA) was added to the drinking water. Mouse terminal surgeries were performed after intraperitoneal injection of avertin (250 mg/kg). Proper analgesia was evaluated by palpebral reflex and toe pinch reflex. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The approval was granted by the Institutional Animal Care and Use Committees of National Cheng Kung University.

Quantitative real-time polymerase chain reaction

Collected hearts and kidneys were stored in RNALater (Ambion, Austin, TX, USA), and
total RNA was extracted with REzol (Protech Technology, Mukilteo, WA, USA). Samples of mRNA were analyzed with SYBR Green-based real-time quantitative RT-PCR (Applied Biosystems, Foster City, CA, USA), with Gapdh as the reference gene in each reaction. Sequences of the primers used for RT-PCR assays are shown in Table S1.

**Western blotting**

Total protein (20 μg) from lower portion of hearts was separated by SDS-PAGE, transferred to PVDF membranes (Pall Gelman Laboratory, Ann arbor, MI, USA) and probed with antibodies recognizing phospho-Erk1/2, Erk1/2, phospho-p38, p38, phospho-JNK, JNK, phospho-Akt, Akt, phospho-GSK3α/β, GSK3α/β (Cell Signaling Technology, Danvers, MA, USA); phospho-FAK (Invitrogen, Carlsbad, CA, USA); FAK (Proteintech, Chicago, IL, USA); and GAPDH (Millipore, Billerica, MA, USA). Immunoreactive proteins were detected using an enhanced chemiluminescence Western blotting detection system (GE Healthcare, Waukesha, WI, USA).

**Immunohistochemical staining**

Formalin-fixed paraffin-embedded tissue sections (5 μm thick) were deparaffinized, blocked, and incubated overnight with primary antibodies against phospho-Erk1/2 (1:500) (Cell Signaling Technology). Secondary antibody staining was done using a kit (VECTASTAIN ABC
kit; Vector Laboratories, Burlingame, CA, USA) and detected with 3, 3′-diaminobenzidine (DAB) substrate-chromogen solution (Dako, Glostrup, Denmark). The slides were counterstained with hematoxylin.

**Data analysis**

Values are shown as mean ± SEM. Statistical analyses were conducted by two-way ANOVA with genotype and treatment as factors. Student’s *t* test was used for comparisons between groups within each experiment. The differences were defined to be statistically significant at *P* < 0.05.

An expanded Methods section appears in the Supplemental Material.
Results

Furo releases Rosi-induced volume expansion and cardiac hypertrophy.

Treatment of Rosi for 4 weeks significantly induced hemodilution, reflected by decreased hematocrit, and cardiac hypertrophy in a dose-dependent manner (Figure S1). Because treatment of Rosi at 10 mg/kg/day for 4 weeks induced a significant increase in heart-to-body weight ratio (HW/BW), we then chose this dosage for the following experiments.

To study the role of plasma volume in Rosi-induced cardiac hypertrophy, we used the diuretic Furo for releasing plasma volume expansion. Neither body weight nor heart rate was significantly changed in Rosi- and Rosi+Furo-treated mice, compared with those in control mice (Table S2). Effects of Rosi, such as decreases in gonadal-to-inguinal fat weight (G/I) ratio, plasma insulin and glucose levels, HOMA index, diastolic and systolic blood pressures, and an increase in plasma adiponectin, were not affected by the Rosi+Furo treatment (Table S2). These data suggest that treatment with Furo did not significantly influence the effects of Rosi on glucose homeostasis in mice. However, renal ENaCγ transcript was significantly increased in the inner medullar of Rosi-treated mice compared to control, and Rosi+Furo-treatment further increased the ENaCγ expression (Table S2). Water consumption was not altered in Rosi-treated mice, but was dramatically increased in Rosi+Furo-treated mice, revealing the effect of Furo on
thirst resulted from diuresis.

Treatment of Rosi for 4 weeks significantly induced hemodilution, reflected by increased plasma volume and decreased hematocrit, and increased cardiac mass (Figure 1A-D). Co-treatment with Furo attenuated Rosi-induced hemodilution and cardiac hypertrophy. These results directly indicate that releasing plasma volume expansion ameliorates cardiac hypertrophy caused by Rosi treatment. Furthermore, we found a strong inverse correlation between HW/BW and hematocrit ($r = -0.4205; P < 0.0001$) (Figure 1E). This observation further supports the causality between plasma volume and cardiac hypertrophy.

Gross appearance showed that both long and short axes of heart were increased by Rosi, and were blunted by co-treatment with Furo (Figure 1F, upper panels). Dissection of the heart did not reveal the difference in the wall thickness of the left ventricle between groups (data not shown). However, the chamber size of left ventricle appeared larger in Rosi treatment, and reversed in Furo co-treatment (Figure 1F, middle panels). Microscopically, we did not see apparent difference in cardiomyocyte diameters among three groups (Figure 1F, lower panels).

Contractile and natriuretic peptide genes and apoptosis are associated with volume
expansion in Rosi-induced cardiac hypertrophy.

Cardiac hypertrophy and stressed heart are known to associate with the fetal gene reprogramming and cardiomyocyte apoptosis.\textsuperscript{14} Rosi treatment tended to decrease expression of α myosin heavy chain (αMHC) and increase expression of β myosin heavy chain (βMHC) (\textit{Figure 2A}), resulting in a significant increase in βMHC-to-αMHC ratio (\textit{Figure 2B}). Rosi treatment also significantly increased expression of α skeletal actin (αSKA). Co-treatment of Furo reversed the increased βMHC, αSKA, and βMHC-to-αMHC ratio, and decreased αMHC. Similarly, natriuretic peptide genes, such as arterial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), were markedly increased in Rosi treatment, and attenuated in the co-treatment with Furo. Furthermore, the number of apoptotic cells, reflected by the TUNEL stain, was significantly increased in the heart of Rosi-treated mice, and was reversed in the heart of Rosi+Furo-treated mice (\textit{Figure 2C}). These results indicated that expression for contractile proteins and natriuretic peptides, as well as cardiomyocyte apoptosis, are predominantly associated with cardiac hypertrophy caused by Rosi treatment. Releasing volume expansion reverses the fetal gene reprogramming and apoptosis, despite the existence of Rosi.

**Erk1/2 signal is dependent on volume expansion in Rosi-induced cardiac hypertrophy.**

Because MAPKs and FAK are important mediators in cardiac hypertrophy,\textsuperscript{21,22} we analyzed
signaling pathways in relation to volume expansion and cardiac hypertrophy. Rosi induced phosphorylation of FAK, and Rosi+Furo reversed the increase in FAK phosphorylation. In MAPK pathways, Rosi treatment increased phosphorylation of Erk1/2, and co-treatment of Furo ameliorated the increase in Erk1/2 phosphorylation (Figure 3A). In contrast, phosphorylation of other MAPKs, including p38 and JNK, was not affected by Rosi or Rosi+Furo. Moreover, phosphorylation of Akt and GSK3α/β was not changed by Rosi or Rosi+Furo. Immunohistochemical analysis confirmed the nuclear localization of phosphorylated Erk1/2 in cardiomyocytes of Rosi-treated mice, but this was not found in the cells of mice co-treated with Furo (Figure 3B). These results suggest that Erk1/2 is a volume-dependent signal in Rosi-induced cardiac hypertrophy.

Glucose and lipid metabolic genes are not dependent on volume expansion in Rosi-induced cardiac hypertrophy.

In the volume overload-induced hypertrophy, fatty acid oxidative capacity is reduced and the heart shifts to preference of glucose metabolism, resembling the fetal metabolic program.23 Because PPARγ is important in regulation of nutrient utilization, we then asked whether genes related to glucose and lipid metabolism are involved in Rosi-mediated cardiac hypertrophy. Expression of genes for glucose import [glucose transporter 1 (GLUT1) and 4 (GLUT4)] and
glycolysis [hexokinase 2 (HK2) and phosphoglycerate kinase 1 (PGK1)] was not affected by Rosi or Rosi+Furo (Figure 4A and S2A). Similarly, expression of genes for glycogenosis and glycogenolysis was not changed among three groups (Figure S2B). However, pyruvate dehydrogenase kinase 4 (PDK4), which inhibits glucose utilization by reducing conversion of pyruvate into acetyl-CoA, was dramatically down-regulated by Rosi (Figure 4A). Interestingly, normalization of cardiac hypertrophy by co-treatment with Furo did not reverse Rosi-induced PDK4 down-regulation.

In the genes involved in cellular fatty acid transport, expression of lipoprotein lipase (LPL) and fatty acid-binding protein 3 (FABP3) was not largely affected by Rosi and Rosi+Furo (Figure 4B). While fatty acid translocase (FAS/CD36) was up-regulated, fatty acid transporter 1 (FATP1) was down-regulated in both Rosi and Rosi+Furo groups. These results suggest that the net lipid transport in the heart might not be largely affected. Surprisingly, the genes involved in mitochondrial fatty acid oxidation, including carnitine palmitoyltransferase 1b and 2 (CPT1b and CPT2) and long chain and medium chain acyl-CoA dehydrogenase (LCAD and MCAD), and mitochondrial uncoupling protein 3 (UCP3) were significantly down-regulated by Rosi treatment (Figure 4C). Co-treatment of Furo did not attenuate Rosi-induced down-regulation of these genes. Moreover, the down-regulation of fatty acid oxidation appears specific to mitochondria
because expression of peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) was not affected by Rosi or Rosi+Furo. Thus, our results suggest that Rosi-induced cardiac hypertrophy is associated with the impairment of mitochondrial fatty acid oxidation. However, amelioration of plasma overload and cardiac hypertrophy does not influence the impaired mitochondrial fatty acid oxidation. These results suggest that Rosi treatment shifts metabolic genes toward the preference of glucose usage, regardless the existence of volume expansion.

PPARγ activation channels a greater portion of lipid to adipose tissue. This action could reduce lipid utilization by the heart, and may be related to the change of abovementioned genes. We found that heart triglyceride content was significantly decreased by Rosi treatment (Figure 4D). This is associated with a significant increase in the subcutaneous inguinal fat weight, but no change in the intraperitoneal gonadal fat weight (Table S2). Consistent with the metabolic gene expression pattern, release of volume overload did not affect heart triglyceride content and inguinal fat weight in the presence of Rosi.

**Pio-induced cardiac hypertrophy is also mediated through volume expansion.**

While clinical trials showed a less risk of Pio on cardiac events, some reports have indicated the involvement of Pio in cardiac hypertrophy. To examine whether Pio induces
cardiac hypertrophy, we treated mice with various dosages of Pio (from 10 to 80 mg/kg/day) for 4 weeks. Effects of Pio, such as decreases in plasma glucose level and G/I ratio, were exhibited in a dose-dependent manner (Figure S3A-B). Consistently, treatment of Pio for 4 weeks dose-dependently decreased hematocrit, and increased HW/BW (Figure S3C-D), indicating that Pio similarly caused plasma volume expansion and cardiac hypertrophy. We further chose 40 mg/kg/day for the following Pio experiments because of its comparable effects on anti-hyperglycemia and volume expansion with the group treated with Rosi at 10 mg/kg/day. Co-treatment with Furo significantly reversed Pio-mediated decrease in hematocrit and increase in heart mass (Figure S4). These results suggest that plasma volume expansion is also an important factor causing cardiac hypertrophy in the treatment of Pio.

**PPARγ heterozygous knockout does not reverse Rosi-induced volume expansion and cardiac hypertrophy.**

To study whether Rosi-induced volume expansion and cardiac hypertrophy are dependent on PPARγ, mice with PPARγ heterozygous knockout (Pparγ+/−) were used. Rosi treatment decreased hematocrit and increased heart mass in both wild-type and Pparγ+/− mice (Figure 5A-C). These results suggest that Rosi still causes volume expansion and cardiac hypertrophy despite the lack of 50% PPARγ. We next examined expression for fetal reprogramming genes,
including natriuretic peptide, and glucose and lipid metabolism, in the heart. No difference was found in basal expression of these genes between wild-type and $Pparg^{+/\cdot}$ mice. Effects of Rosi on changes in expression of genes for natriuretic peptide (BNP) and metabolism (PDK4 and MCAD) were similar between wild-type and $Pparg^{+/\cdot}$ mice (Figure 5D-F). Together, these results suggest that Rosi can still use the residue 50% PPARγ to mediate the dysregulation of both hypertrophic and metabolic genes in the heart.

**Loss of ligand-binding ability of PPARγ blunts Rosi-induced volume expansion and cardiac hypertrophy.**

We next used mice heterozygous for the ligand-binding defective mutation of PPARγ ($Pparg^{P465L/+}$) to further tested whether Rosi-induced volume expansion and cardiac hypertrophy are dependent on interaction between agonist and PPARγ. Interestingly, effects of Rosi on decrease in hematocrit were significantly blunted in $Pparg^{P465L/+}$ mice (Figure 6A). Consistent with the absence of volume expansion, Rosi treatment did not induce cardiac hypertrophy in $Pparg^{P465L/+}$ mice (Figure 6B-C). No difference was found in basal expression of these genes between wild-type and $Pparg^{P465L/+}$ mice. None of the expression of genes for natriuretic peptide (BNP) and metabolism (PDK4 and MCAD) was changed in Rosi-treated $Pparg^{P465L/+}$ mice (Figure 6D-F). These results suggest that Rosi-induced volume expansion and
cardiac hypertrophy, as well as dysregulations in cardiac hypertrophic and metabolic genes, requires the intact ligand-binding ability of PPARγ.
Discussion

The report of TZDs causing cardiac hypertrophy in rodents can be traced back as early as 1999, in which the authors found that treatment of Rosi at 10 mg/kg/day for 3 weeks caused cardiac hypertrophy and hemodilution in rats.\textsuperscript{12} While it has been thought that plasma volume expansion is the cause of TZD-induced cardiac hypertrophy, other reports showed that TZDs prevent pressure or angiotensin II-induced cardiac hypertrophy in rodents.\textsuperscript{4, 5} The inconsistency of TZD on cardiac hypertrophy suggests a pleiotropic nature of PPAR\(\gamma\) activation in the heart. Several genetic studies demonstrated that cardiac-specific PPAR\(\gamma\) knockout induced spontaneous cardiac hypertrophy,\textsuperscript{8-10} suggesting that cardiac PPAR\(\gamma\) is beneficial in maintenance of heart homeostasis. In addition, PPAR\(\gamma\) heterozygous knockout increased the susceptibility to pressure-induced cardiac hypertrophy.\textsuperscript{5} In contrast, other reports showed evidence supporting the detrimental role of PPAR\(\gamma\) in development of cardiac hypertrophy. Overexpression of PPAR\(\gamma\) in cardiomyocytes causes cardiac hypertrophy. Treatment of Rosi at 10 mg/kg/day for 2 weeks in mice with cardiac-specific overexpression of PPAR\(\gamma\) led to further deterioration of cardiac hypertrophy.\textsuperscript{13} Thus, the bona fide nature of PPAR\(\gamma\) and its agonist in cardiac function remained inconclusive.

It has been suggested that both cardiac and non-cardiac PPAR\(\gamma\) contribute to TZD-induced
cardiac hypertrophy. In the study by Duan et al., a treatment of cardiac-specific PPARγ knockout mice with Rosi at 10 mg/kg/day for 4 weeks caused, although to a lesser extent to Rosi-treated wild-type mice, cardiac hypertrophy. However, another study by Caglayan et al. showed the negative effect of Pio (0.2% (about 300 mg/kg/day) for 6 weeks) on cardiac hypertrophy in cardiac-specific PPARγ knockout mice. For the role of PPARγ in the non-cardiac tissues, two studies demonstrated that renal PPARγ activation by TZDs up-regulates ENaCγ and increases water reabsorption. While TZD-induced hemodilution occurred only in the presence of intact collecting duct PPARγ, effects of collecting duct PPARγ on TZD-induced cardiac hypertrophy were not addressed in their studies. Thus, no direct evidence demonstrates non-cardiac PPARγ as the cause of TZD-induced cardiac hypertrophy.

The mechanism of TZD in increase of insulin sensitivity is redirection of lipid to the adipose tissues, which is orchestrated by adipose PPARγ with coordination of non-adipose PPARγ. Thus, the in vivo effects of TZDs on the heart are complicated by changes in plasma and tissue lipid metabolism. Our current work, together with reports by others, clearly demonstrates a paradoxical reduction in rodents of the cardiac expression of many usual PPARγ downstream genes, including those for lipid transport and fatty acid oxidation. Similarly, we also found that Rosi treatment led to the preference of nutrient usage from fatty acids to
carbohydrates in the heart. This is coordinated by down-regulation of genes for glycolysis inhibition (PDK4) and mitochondrial fatty acid oxidation in the heart. Redirection of lipid to the adipose tissue also can be evidenced by reduction of heart triglyceride content and increase of subcutaneous inguinal fat weight. Importantly, the shift of energy preference to glucose in the heart and redirection of lipid to adipose tissue, are not affected by the release of volume overload. Thus, the action of TZD on either cardiac PPAR\(\gamma\) activation or whole body lipid mobilization is not likely the cause of TZD-induced cardiac hypertrophy.

Although the pharmacologic agent TZD has been suggested to exert PPAR\(\gamma\)-independent effects,\(^{25}\) PPAR\(\gamma\) activation with several non-TZD class compounds, such as X334 and COOH, also resulted in cardiac hypertrophy.\(^{26,27}\) These results support the biological action of PPAR\(\gamma\) on cardiac hypertrophy despite classification of agonists. Although the genetic model with cardiac PPAR\(\gamma\) over-expression induced cardiac hypertrophy,\(^{13}\) this model does not completely mirror the TZD-mediated condition. Another approach to address PPAR\(\gamma\) dependence in TZD-induced cardiac hypertrophy would be the use of PPAR\(\gamma\) deficient mice. However, in the study of Asakawa et al., treatment of Pio (0.01%; about 15 mg/kg/day) for 4 weeks did not elicit cardiac hypertrophy in wild-type or heterozygous PPAR\(\gamma\) knockout mice.\(^5\) In our study, we analyzed the effect of Rosi on cardiac hypertrophy in both heterozygous knockout (\(Pparg^{+/}\)) and
ligand-binding defective ($Pparg^{P465L/+}$) mice. Interestingly, $Pparg^{P465L/+}$, but not $Pparg^{+/+}$, mice display the ability to attenuate Rosi-induced plasma volume expansion and cardiac hypertrophy. These results indicate that PPARγ heterozygous knockout is not sufficient to attenuate Rosi-induced cardiac hypertrophy. Lack of intact ligand binding or dominant negative effect of $Pparg^{P465L/+}$ mice causes attenuation of Rosi-induced cardiac hypertrophy. Nevertheless, appearance of cardiac hypertrophy in $Pparg^{P465L/+}$ and $Pparg^{+/+}$ mice was tightly associated with existence of plasma volume expansion, confirming the dependence of Rosi-induced cardiac hypertrophy on plasma volume expansion.

Volume overload induces eccentric cardiac hypertrophy, in which chamber volume enlarges without a relative increase or even with a relative decrease in its wall thickness.²⁸ Signaling molecules, such as Erk1/2, Akt, GSK3α/β and p70s6k, were involved in volume overload-induced cardiac hypertrophy.²⁹ Our data showed that Erk1/2 was activated in Rosi-induced cardiac hypertrophy. Release volume overload reversed Rosi-induced Erk1/2 activation. Moreover, stretch-induced FAK activation is shown to activate Erk1/2.²² Consistently, we found that Rosi-induced Erk1/2 activation is associated with FAK activation, which is reversed by the release of volume overload. Moreover, the increased phosphorylation of Erk1/2 and no change in phosphorylation of Akt, GSK3α/β, and JNK by Rosi treatment in our model are
consistent with others. However, we did not observe the change in phosphorylation of p38. Nevertheless, these results suggest that FAK-Erk1/2 activation is critical in TZD-induced volume overload and cardiac hypertrophy.

Volume overload-mediated cardiac hypertrophy is also associated with the reprogramming of cardiac fetal genes, with increased expression of genes for natriuretic peptides (ANP, BNP) and contractile proteins (αSKA), and decreased expression of genes for lipid oxidation (MCAD). In our results, we found cardiac genes encoding natriuretic peptides, contractile proteins, and glucose/lipid metabolism enzymes were altered under Rosi treatment. However, only genes for natriuretic peptides and contractile proteins, but not glucose/lipid metabolism enzymes, were reversed by the release of volume overload. Thus, Rosi-mediated changes on expression of genes for contractile proteins and natriuretic peptides are dependent on volume overload, whereas changes on expression of genes for glucose/lipid metabolism are independent on volume overload.

Although it has been shown that Pio is associated with a reduced risk of overall mortality and congestive heart failure compared with Rosi, some studies demonstrated elevated risks on cardiac events of Pio. For example, a meta-analysis demonstrated a slightly greater increase in
risk of heart failure with Rosi (2.2-fold) than with Pio (1.3-fold), although the between treatment differences were not statistically significant. Another study also reported an increase in the risk of heart failure (1.4-fold) but not ischemia cardiovascular outcomes with Pio. In animal models, while studies indicate that Pio induced cardiac hypertrophy, others showed the negative effect of Pio treatment on cardiac hypertrophy. In this study, we found that Pio induced hemodilution as low as 10 mg/kg/day and cardiac hypertrophy at 40 mg/kg/day. Furthermore, we found that Pio-induced cardiac hypertrophy was reversed by co-treatment with Furo, indicating that Pio-induced cardiac hypertrophy is also mediated through volume expansion. Thus, caution is urged for the use of Pio in patients with signs and symptoms of suggestive heart failure.

In summary, we found that releasing volume overload attenuated Rosi-induced cardiac hypertrophy, hypertrophic gene reprogramming, cardiomyocyte apoptosis, and hypertrophy-related signal activation. These factors seem to be the “on-targets” of volume expansion despite the existence of Rosi. In contrast, glucose and lipid metabolism genes in the heart were altered by Rosi, but not reversed by the release of volume overload. Thus, nutrient metabolism genes are likely the “off-targets” of volume expansion, and are dependent on the existence of Rosi. Finally, Rosi-induced volume expansion and cardiac hypertrophy, as well as dysregulations in hypertrophic and metabolic genes in the heart, requires the intact
ligand-binding ability of PPARγ. Our results also provide the direct evidence that co-treatment of
the diuretic Furo is able to lower side effects of TZD-induced volume expansion and
cardiovascular events. This is a strategy that could adopt immediately without withdrawal of
TZDs and test of new drug targeting PPARγ.
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Conflict Interest

None declared
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Figure Legends

Figure 1

Effects of Furo on Rosi-induced volume expansion and cardiac hypertrophy. (A) Plasma volume expressed as a ratio to body weight, (B) hematocrit, (C) heart weight, and (D) the heart-to-body weight ratio (HW/BW) of control (black column), Rosi-treated (white column), and Rosi+Furo-treated mice (gray column). Numbers inside columns = \( n \) in each group. *\( P < 0.05; \) **\( P < 0.01; \) and ***\( P < 0.001 \) versus control mice. ##\( P < 0.01 \) and ###\( P < 0.001 \) versus Rosi-treated mice. (E) Correlation between the HW/BW ratio and hematocrit in control (■), Rosi-treated (○), and Rosi+Furo-treated mice (×). \( R = -0.4205; \) \( P < 0.0001 \). (F) Gross appearance (upper panels), cross section (middle panels) and microscopic examination (lower panels) of the heart. The scale bar is 50 μm.

Figure 2

Expression of contractile and natriuretic peptide genes and apoptosis in Rosi-induced cardiac hypertrophy. (A) Expression of contractile and natriuretic peptide genes, (B) the ratio of \( \beta \)MHC to \( \alpha \)MHC, (C) the number of TUNEL positive cells in whole section of the heart of control (black column), Rosi-treated (white column), and Rosi+Furo-treated mice (gray column). mRNA amount is expressed relative to the average expression in control mice. \( n = 6 \) in each group. *\( P <
0.05, **P < 0.01 and ***P < 0.001 versus control mice. #P < 0.05, ##P < 0.01, and ###P < 0.001 versus Rosi-treated mice.

**Figure 3**

Signaling pathways in Rosi-induced cardiac hypertrophy. (A) Immunoblot analyses on phosphorylation of FAK, Erk1/2, p38, JNK, Akt, and GSK3α/β in the heart. Each band represents a tissue extract from a single mouse. (B) Immunohistochemical staining of phosphorylated Erk1/2 in the heart. Cells expressing phosphorylated Erk1/2 are stained brown. The inset shows a higher magnification of the arrow-pointed cell. The scale bar is 50 μm.

**Figure 4**

Expression of glucose and lipid metabolism genes in Rosi-induced cardiac hypertrophy. Expression of genes for (A) glucose utilization, (B) lipid transport, (C) fatty acid oxidation, and (D) triglyceride (TG) contents in the heart of control (black column), Rosi-treated (white column), and Rosi+Furo-treated mice (gray column). mRNA amount is expressed relative to the average expression in control mice. n = 12 in each group. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control mice. #P < 0.05 versus Rosi-treated mice.
Figure 5

Plasma volume expansion and cardiac hypertrophy in Rosi-treated \( Pparg^{+/-} \) mice. (A) Hematocrit, (B) heart weight, and (C) HW/BW of \( Pparg^{+/+} \) and \( Pparg^{+/-} \) mice. (D-F) Expression of hypertrophic and metabolism genes in the heart of \( Pparg^{+/+} \) and \( Pparg^{+/-} \) mice. mRNA amount is expressed relative to the average expression in \( Pparg^{+/+} \) mice without Rosi treatment. The mice treated without (black column) or with Rosi (white column) are shown. Numbers inside columns = \( n \) in each group. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \) against its control mice.

Figure 6

Attenuation of plasma volume expansion and cardiac hypertrophy in Rosi-treated \( Pparg^{P465L/+} \) mice. (A) Hematocrit, (B) heart weight, (C) HW/BW of \( Pparg^{+/+} \) and \( Pparg^{P465L/+} \) mice. (D-F) Expression of hypertrophic and metabolism genes in the heart of \( Pparg^{+/+} \) and \( Pparg^{P465L/+} \) mice. mRNA amount is expressed relative to the average expression in \( Pparg^{+/+} \) mice without Rosi treatment. The mice treated without (black column) or with Rosi (white column) are shown. Numbers inside columns = \( n \) in each group. *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \) against its control mice. No significant (N.S.) difference was found between control and Rosi-treated \( Pparg^{P465L/+} \) mice.