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Treatment with Treprostinil and Metformin Normalizes Hyperglycemia and Improves Cardiac Function in Pulmonary Hypertension Associated with Heart Failure with Preserved Ejection Fraction (PH-HFpEF)

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Abstract

Objective: Pulmonary hypertension due to left heart disease (PH-LHD; Group 2), especially in the setting of heart failure with preserved ejection fraction (HFpEF), is the most common cause of

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Disclosures

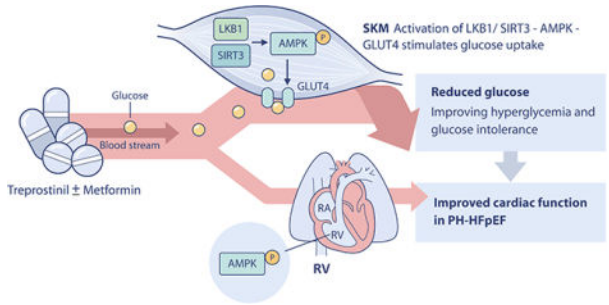
Dr. Gladwin is a co-inventor of pending patent applications and planned patents directed to the use of recombinant neuroglobin and heme-based molecules as antidotes for CO poisoning, which have been licensed by Globin Solutions, Inc. Globin Solutions, Inc. also has an option to a potential therapeutic for CO poisoning from VCU, hydroxycobalamin. Dr. Gladwin is a shareholder, advisor, and director in Globin Solutions, Inc. Dr. Gladwin is also co-inventor on patents directed to the use of nitrite salts in cardiovascular diseases, which were previously licensed to United Therapeutics and Hope Pharmaceuticals, and is now licensed to Globin Solutions. Additionally, Dr. Gladwin is a co-investigator in a research collaboration with Bayer Pharmaceuticals to evaluate riociguat as a treatment for patients with sickle cell disease, and is a consultant for Bayer and Complexa pharmaceuticals. The other authors report no conflicts.

PH worldwide, however, at present, there is no proven effective therapy available for its treatment. PH-HFpEF is associated with insulin resistance and features of metabolic syndrome. The stable prostacyclin analog, treprostinil, is an effective and widely used FDA-approved drug for the treatment of pulmonary arterial hypertension. While the effect of treprostinil on metabolic syndrome is unknown, a recent study suggests that the prostacyclin analog beraprost can improve glucose intolerance and insulin sensitivity. We sought to evaluate the effectiveness of treprostinil in the treatment of metabolic syndrome-associated PH-HFpEF.

Approach and Results: Treprostinil treatment was given to mice with mild metabolic syndrome-associated PH-HFpEF induced by high-fat diet (HFD) and to SU5416/Obese ZSF1 rats, a model created by the treatment of rats with a more profound metabolic syndrome due to double leptin receptor defect (obese ZSF1) with a vascular endothelial growth factor receptor blocker SU5416. In HFD-exposed mice, chronic treatment with treprostinil reduced hyperglycemia and pulmonary hypertension. In SU5416/Obese ZSF1 rats, treprostinil improved hyperglycemia with similar efficacy to that of metformin (a first-line drug for type 2 diabetes); the glucose-lowering effect of treprostinil was further potentiated by the combined treatment with metformin. Early treatment with treprostinil in SU5416/Obese ZSF1 rats lowered pulmonary pressures, and a late treatment with treprostinil together with metformin improved pulmonary artery acceleration time to ejection time ratio (PAAT/ET) and tricuspid annular plane systolic excursion (TAPSE) with AMP-activated protein kinase (AMPK) activation in skeletal muscle and the right ventricle.

Conclusions: Our data suggest a potential use of treprostinil as an early treatment for mild metabolic syndrome-associated PH-HFpEF and that combined treatment with treprostinil and metformin may improve hyperglycemia and cardiac function in a more severe disease.

Graphical Abstract



Keywords

Pulmonary Hypertension; Metabolic syndrome; HFpEF; Treprostinil; Metformin

Introduction

Pulmonary hypertension due to left heart disease (PH-LHD; Group 2), especially in the setting of heart failure with preserved ejection fraction (HFpEF), is among the most frequent causes of pulmonary hypertension worldwide. Features of metabolic syndrome, including obesity, diabetes, hyperlipidemia, and hypertension, are recognized as risk factors for developing PH-HFpEF.[1–3] In fact, two or more of these features are commonly seen in

patients with PH-HFpEF.[4] Although the exact prevalence varies based on definitions and diagnostic methods, the range in the reported occurrence of pulmonary hypertension (simply defined as having a mean pulmonary artery pressure greater than or equal to 25 mm Hg) is 23%−83% in patients with HFpEF.[5–7] A large recent retrospective analysis of patients with right heart catheterization suggested that using more stringent values for high intrinsic pulmonary vascular resistance, such as a transpulmonary gradient > 12 mm Hg, a pulmonary vascular resistance > 3 woods units, or a diastolic pressure gradient > 7 mm Hg, was present in 48.9%, 34.2%, and 13.7% of patients with HFpEF, respectively.[7–9] Patients with PH-HFpEF develop more severe symptoms than those with HFpEF and suffer significant exercise intolerance, frequent hospitalization and reduced survival.[1] At present, there are no approved specific therapies for PH-HFpEF. Most drugs which target only the pulmonary vasculature tested thus far have failed to demonstrate any significant benefit in the treatment of PH-HFpEF.[10–14]

Our group has recently developed a two-hit model of PH-HFpEF, which combines endothelial injury using the VEGF receptor blocker SU5416 in rats with multiple features of metabolic syndrome due to double leptin receptor defects (obese ZSF1).[15] Clinical features seen in PH-HFpEF patients, including elevated left ventricular (LV) end-diastolic pressure, right ventricular (RV) systolic pressure and right atrial pressure, preserved LV ejection fraction, as well as biventricular hypertrophy, are consistently observed in this model.[15] In addition, SU5416/obese ZSF1 rats develop impaired insulin sensitivity characterized by high fasting glucose levels, elevated glycated hemoglobin (HbA1c) and defective glucose tolerance. Using this model, we have reported that drugs which target both cardiopulmonary pathophysiological defects and metabolic syndrome, such as metformin and nitrite, a drug that is metabolized to nitric oxide, reduce pulmonary pressures and vascular remodeling, and improve glucose intolerance, glucose uptake, and insulin resistance.[15] Moreover, metformin has also been recently shown to improve metabolic syndrome-associated PH and/or HFpEF in preclinical models induced by high-fat diet (HFD) alone or in combination with supra-coronary aortic banding and the antidepressant olanzapine.[16, 17]

The prostacyclin analog, treprostinil, is one of the US Food and Drug Administration-approved drugs that consistently leads to hemodynamic improvement of pulmonary arterial hypertension (PAH, Group 1).[18] While the effect of treprostinil on metabolic syndrome is unknown, a prostacyclin analog, beraprost sodium, has been recently shown to reverse features of metabolic syndrome in obese Zucker rats.[19] In addition, prostacyclin precursor L-carnitine has been shown to prevent the development of HFpEF in rats fed with a high-salt diet.[20] Still, the mechanism behind these observations remains elusive. Due to a potential ability of treprostinil to regulate both systemic metabolic defects and pulmonary vascular disease, in the present work we sought to evaluate the effectiveness and mechanism of treprostinil in the treatment of metabolic syndrome-associated PH-HFpEF.

Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh and the Indiana University School of Medicine. All the experiments were blindly performed and analyzed. Detailed methods and Major

Resources Table are available in the Supplemental Materials. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animal studies

The PH-HFpEF models were developed as described before.[15, 21, 22] For the rat model, we used eight-week old male obese ZSF1 rats, which present with multiple features of metabolic syndrome, diabetic nephropathy and diastolic dysfunction,[23, 24] as well as their lean littermates (Charles River, Wilmington, MA). VEGFR2 antagonist SU5416 (Sigma-Aldrich, St. Louis, MO), which induces lung endothelial injury and/or apoptosis,[25] was dissolved in CMC buffer (0.5 % sodium carboxymethyl cellulose, 0.9 % sodium chloride, 0.4 % polysorbate 80, and 0.9 % benzyl alcohol) and given as a single 100 mg/kg subcutaneous injection to obese and/or lean ZSF1 rats at day 0. As female obese ZSF1 rats are resistant to the development of diabetes (hyperglycemia) and renal disease (proteinuria), [26] only male rats were used in this study. For the mouse model, eight-week old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were fed a regular diet (RD; 15% lipids/kcals) or HFD (60% lipids/kcals; Research Diets, New Brunswick, NJ) for 16 weeks. Treprostinil (40, 300 and 900 ng/kg/min; United Therapeutics Corporation, Silver Spring, MD) was delivered through osmotic minipumps (model 2ML4 for rats; model 2006 for mice; duration of 4 or 6 weeks, respectively; Alzet, Cupertino, CA). After 28 or 42 days, used minipumps were replaced with fresh minipumps. Maximum of 3 implantations were performed in each animal. Metformin (300 mg/kg/day; Spectrum Chemical, New Brunswick, NJ) was given in drinking water. While this dosage of metformin is higher than the oral dose of metformin (~30 mg/kg/day) given to patients with type 2 diabetes, it is considered to be clinically relevant as therapeutic effects of 300 mg/kg/day metformin in rats are similar to the effects of 30 mg/kg/day of metformin in humans.[27, 28] All animals were maintained in a normoxic environment.

Hemodynamic and ventricular measurements

In brief, mice and rats were anesthetized with isoflurane (1–2 % v/v). The trachea was cannulated, and rats were ventilated at the rate of 75 to 80 bpm with a tidal volume of 2.5 mL. RV systolic pressure, LV end-diastolic pressure and LV ejection fraction were measured using an admittance pressure-volume catheter. Weights of RV and LV+septum were normalized to tibial length as indexes of ventricular mass.

Transthoracic echocardiography

Transthoracic echocardiography was performed and analyzed using the Vevo 770 System (VisualSonics, ON, Canada). Rats were induced into anesthesia for handling with ~3 % v/v isoflurane and maintained with ~1.5 % v/v. The end-diastolic thickness of left ventricular posterior wall and intraventricular septum, early diastolic mitral annular velocity, mitral peak velocity of late filling, pulmonary artery acceleration time, ejection time, tricuspid annular plane systolic excursion and right ventricular dimension at end-diastole were measured.

Statistical Analysis

Statistical analyses were performed using Prism 8.2.0 software (La Jolla, CA). Statistical comparison between two groups for *in vitro* studies were performed using the unpaired Student's *t*-test after testing for normality (Shapiro-Wilk test; assuming equal variance). Comparison among ≥ 3 groups was performed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test if the data followed a normal distribution, otherwise nonparametric Kruskal-Wallis test with Dunn's *post hoc* analysis was used. For differences in blood glucose levels during the glucose tolerance test, two-way ANOVA followed by Bonferroni's *post hoc* test was performed. A limitation of the study is a small sample size in some experiments, in which only 3 animals were used. Values of $P < 0.05$ were considered statistically significant.

Results

Chronic treprostinil administration improves hyperglycemia and pulmonary hypertension in HFD-exposed mice

To evaluate the preventative effect of treprostinil on metabolic syndrome-associated PH-HFpEF, treprostinil (40 ng/kg/min, the effective dose for PAH therapy with similar average infusion rate achieved in clinical trials, on a ng/m^2 basis) was given through osmotic minipumps for 16 weeks in mice fed with a HFD (Figure 1A), which has been shown to induce PH and/or HFpEF phenotype in mice.[16, 21, 22, 29, 30] Consistent with previous studies, HFD-exposed mice had significantly higher body weights, hyperglycemia and glucose intolerance when compared to RD-treated mice (Figure 1B through 1D). Additionally, HFD-exposed mice developed mild PH and/or HFpEF phenotype as evidenced by elevated right ventricular systolic pressure (RVSP), higher left ventricular end diastolic pressure (LVEDP), preserved left ventricular ejection fraction (LVEF) and both LV and RV hypertrophy (Figure 1E through 1I). In contrast, chronic treprostinil treatment significantly lowered HbA1c levels and improved glucose intolerance independent of changes in body weight (Figure 1B through 1D). Furthermore, a tendency for treprostinil to lower pulmonary pressures was observed in HFD-exposed mice ($P = 0.058$; Figure 1E), although treprostinil caused no significant differences in LVEDP and biventricular hypertrophy (Figure 1F, 1H and 1I). Collectively, these data demonstrate that chronic treprostinil treatment improves glucose metabolism and lowers pulmonary hypertension in a mild mouse model of PH-HFpEF induced by HFD.

Early treatment with treprostinil reduces hyperglycemia, improves lipemia and lowers pulmonary pressures in SU5416/obese ZSF1 rats

We next evaluated the early treatment effect of treprostinil in rats with a more profound metabolic syndrome-associated with PH-HFpEF. Treprostinil (40, 300 and 900 ng/kg/min) was given through osmotic minipumps concurrently with SU5416 exposure to 8-week old obese ZSF1 rats (day 0, Figure 2A and Supplemental Figure IA) for 14 weeks. Note that these rats already had insulin resistance and glucose intolerance at this time point, but have not yet developed HFpEF and/or PH phenotypes.[24] The effect of treprostinil on PH-HFpEF was compared to that of metformin (300 mg/kg), the first-line drug for type 2 diabetes, as well as a newly identified early intervention in this PH-HFpEF model.[15] While the low dose

of treprostinil (40 ng/kg/min) was found to be effective in reducing hyperglycemia and pulmonary pressures in the mild mouse model of metabolic syndrome-associated PH-HFpEF induced by HFD (Fig 1), no significant effect of 40 ng/kg/min of treprostinil on hyperglycemia and pulmonary pressures was observed in Ob-Su rats (Supplemental Figure I). On the other hand, moderate dose of treprostinil (300 ng/kg/min) significantly reduced fasting blood glucose levels with similar efficacy to that of metformin, independent of changes in body weight (Figure 2B and 2C). A dose of 300 ng/kg/min of treprostinil treatment also significantly lowered HbA1c levels and improved glucose intolerance compared to the untreated Ob-Su animals (Figure 2D and 2E). Plasma obtained from Ob-Su rats was noted to be lipemic and milky (lactescent), and this phenomenon was improved by treprostinil treatment (Figure 2F). Additionally, treprostinil (300 ng/kg/min) treatment resulted in lower right ventricular systolic pressure compared to the untreated Ob-Su rats (Figure 2G), although no effect on medial wall thickness was observed (Supplemental Figure II). Similar effects of treprostinil on improving hyperglycemia and lipemia was observed with the higher dose (900 ng/kg/min), but not the lower dose (40 ng/kg/min) of treprostinil in Ob-Su rats (Supplemental Figure IB through IE). Collectively, our results demonstrate that a treatment with moderate dose of treprostinil can lead to a beneficial effect on glucose metabolism with efficacy similar to that of metformin. While an infusion rate of 300 ng/kg/min of treprostinil in Ob-Su rats is 8 times the average rate used in PAH clinical trials, this dose significantly improves hyperglycemia and pulmonary hypertension in Ob-Su rat model of metabolic syndrome-associated PH-HFpEF. As patients with PH-HFpEF usually present with a higher body mass index (42.1 ± 10.1) compared to that of patients with PAH (30.7 ± 5.7),[46] it is possible that a higher dose (infusion rate) of treprostinil may be needed for the treatment of PH-HFpEF. Since metformin shows a better efficacy with respect to lowering pulmonary pressures than does treprostinil and has been shown to reverse PH-HFpEF via inhibiting IL6/STAT3-mediated pulmonary vascular remodeling,[17] these data suggest that a combination therapy of treprostinil and metformin may be beneficial for the late treatment of metabolic syndrome-associated PH-HFpEF.

SU5416/obese ZSF1 rats progressively develop metabolic syndrome-associated PH-HFpEF

In order to assess disease progression and to provide baseline features for the late treatment study in SU5416/obese ZSF1 rats, we monitored echocardiographic, metabolic and hemodynamic changes after 7 and 14 weeks of SU5416 administration in obese ZSF1 rats. Echocardiographic findings in SU5416-exposed obese ZSF1 rats showed a time-independent increase in left ventricular posterior wall (LVPW) and interventricular septum (IVS) thickness and decrease in the ratio of early diastolic mitral annular velocity to mitral peak velocity of late filling (E/A), which reflects grade I diastolic dysfunction, compared to lean rats throughout the entire study (Figure 3A through 3D). In line with progressive decrease in pulmonary artery acceleration time to ejection time ratio (PAAT/ET) and tricuspid annular plane systolic excursion (TAPSE), the latter of which reflects reduced RV systolic function, together with increased RV diastolic dimension (RVDd), SU5416/obese ZSF1 rats also exhibited elevated RV systolic pressure, fasting blood glucose and HbA1c levels in a time-dependent manner (Figure 3E through 3G; Supplemental Figure III). Note that SU5416 administration alone in lean rats had no effect on the echocardiographic, metabolic and hemodynamic features of metabolic syndrome-associated PH-HFpEF compared to untreated

lean animals (Fig 3 and Supplemental Figure III). We also note that obese ZSF1 rats do not develop resting PH (~ 27.5 mm Hg vs. 25 mmHg in lean rats; P = NS; data not shown). Together, these results demonstrate that SU5416/obese ZSF1 rats progressively develop metabolic syndrome-associated PH-HFpEF.

Late treatment with treprostinil and metformin normalizes hyperglycemia and improves cardiac function in SU5416/obese ZSF1 rats

To evaluate the late treatment effect of treprostinil, alone or in combination with metformin, treprostinil (300 ng/kg/min) and metformin (300 mg/kg) were given 7 weeks after a single injection of SU5416 to obese ZSF1 rats (Figure 4A). Note that these rats already exhibited mild LV diastolic dysfunction accompanied by more profound hyperglycemia and elevated right ventricular systolic pressure at this time point (Figure 3; Supplemental Figure III). Late treatment with treprostinil significantly lowered HbA1c levels and improved glucose intolerance independent of changes in body weight. This was further potentiated by the combined treatment with metformin in more severely affected SU5416/obese ZSF1 rats (Figure 4B through 4D). Additionally, relatively mild lipemia was observed in Ob-Su rats treated with treprostinil alone or in combination with metformin (Supplemental Figure IV). While the late treatment with treprostinil alone or in combination with metformin failed to reverse the increased right ventricular systolic pressure and had no effect on E/A, biventricular hypertrophy, LVPW, LV EF and cardiac output (Figure 4E and 4F; Supplemental Figure V), our data showed that the combination of treprostinil and metformin significantly increased PAAT/ET and TAPSE relative to the untreated, but more severely affected Ob-Su animals (Figure 4G and 4H). Together, these data demonstrate the benefits of a late treatment with treprostinil specifically related to improving hyperglycemia and glucose intolerance. These data also suggest that combination treatment with treprostinil and metformin may improve cardiac function in metabolic syndrome-associated PH-HFpEF.

Treatment with treprostinil and metformin improves metabolic syndrome-associated PH-HFpEF via skeletal muscle activation of AMPK-GLUT4 and systemic improvement of insulin resistance

To determine whether the beneficial effect of treprostinil on improving hyperglycemia and lipemia in Ob-Su rats is related to changes in insulin secretion and lipid contents, plasma insulin, cholesterol and triglyceride levels were measured. Our data showed that late treatment with treprostinil alone in our severe model had no effect on plasma insulin, cholesterol and triglyceride levels in Ob-Su rats compared to the untreated animals (Figure 5A through 5C), however, combination treatment with treprostinil and metformin significantly lowered plasma insulin in Ob-Su rats, suggesting improved insulin resistance (Figure 5A).

To further evaluate the mechanism by which treprostinil improves glucose metabolism, we measured AMPK levels in liver and skeletal muscle, the main organs for glucose metabolism through inhibition of gluconeogenesis and stimulation of glucose uptake, respectively. As shown in Supplememtal Figure VIA, phosphorylation of AMPK was not altered in liver of treprostinil-treated Ob-Su rats compared to the untreated animals, suggesting the glucose-lowering effect of treprostinil is independent of suppression of hepatic glucose production.

On the other hand, our data showed a significant increase in AMPK phosphorylation in skeletal muscle of treprostinil-treated Ob-Su rats, accompanied by increased membrane translocation of glucose transporter 4 (GLUT4) compared to the untreated animals (Figure 6A and 6B). Together, our data suggest that treprostinil treatment improves hyperglycemia and glucose intolerance via AMPK-GLUT4-mediated glucose uptake in skeletal muscle. Our data also suggest that the combination of treprostinil and metformin improves PH-HFpEF-associated metabolic syndrome via both local (skeletal muscle) and systemic improvement of glucose uptake and insulin resistance.

Treprostinil activates skeletal muscle AMPK-GLUT4 signaling pathway via upstream activators, LKB1 and SIRT3

Given that liver kinase B1 (LKB1), transforming growth factor-β-activated kinase-1 (TAK1), calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) and sirtuin-3 (SIRT3) are known upstream activators of AMPK,[31–34] we next investigated their expression/activation levels in skeletal muscle of treprostinil-treated Ob-Su rats. As shown in Figure 6C, 6D and Supplememtal Figure VIB and VIC, expression levels of CaMKK2 and TAK1 are not altered in the skeletal muscle of treprostinil-treated Ob-Su rats compared to the untreated animals, while the increased LKB1 expression and elevated SIRT3 activation levels (the antibody we used specifically recognizes the short active form of SIRT3 at ≈ 28 kDa, which contains the catalytic domain and regulates deacetylation of downstream substrates) was observed. The same AMPK/LKB1/SIRT3-activating effect was observed when C2C12 skeletal muscle myoblasts were treated with plasma obtained from treprostinil-treated Ob-Su rats (Figure 6E), whereas direct stimulation of treprostinil at relevant concentrations did not result in significant activation of AMPK, LKB1 and SIRT3 (Supplemental Figure VID). A higher ratio of intercellular AMP to ATP levels was also observed in C2C12 skeletal muscle myoblasts stimulated with plasma obtained from treprostinil-treated Ob-Su rats (Figure 6F). Together, these data suggest that treprostinil activates AMPK through upstream regulation of LKB1 and SIRT3, along with a substantial increase in the AMP:ATP ratio. These data also suggest that the metabolite of treprostinil may be required for the activation of LKB1/SIRT3-AMPK signaling pathway.

To probe for further evidence, we examined the effect of siRNA-mediated knockdown of LKB1 and SIRT3 on treprostinil-mediated AMPK activation. C2C12 cells were transiently transfected with siRNA targeting LKB1, SIRT3 or scrambled control for 24 hours before stimulation with plasma obtained from Ob-Su rats, in the presence or absence of treprostinil. As shown in Figure 7, the ability of treprostinil-contained plasma to activate AMPK was reduced in LKB1 and SIRT3 knockdown cells, further suggesting that LKB1 and SIRT3 are required for treprostinil-mediated AMPK activation.

Treatment with treprostinil and metformin activates AMPK in the RV

To understand how late treatment with treprostinil and metformin might improve PAAT/ET and TAPSE (Figure 4), we investigated pulmonary vascular changes, along with AMPK activation in the RV, since AMPK is thought to be a central player in regulating cardiac function, cardiomyocyte contractility and lipid accumulation in the RV.[16, 35] While the late treatment with treprostinil alone or in combination with metformin failed to reverse the

elevated medial wall thickness in the pulmonary arteries (Figure 8A) and had no effect on PDGF-BB-induced proliferation of pulmonary arterial smooth muscle cells (PASMCs; Supplemental Figure VIIB), an increase in AMPK activation levels was observed in the RV of Ob-Su rats treated with treprostinil and metformin (Figure 8B). AMPK has been shown to modulate cardiac contractile function through upstream LKB1 and downstream target Ser150 of cardiac Troponin I.[36–38] However, our data showed that increased AMPK activation by treprostinil and metformin was not accompanied by changes in LKB1 nor was associated with phosphorylation levels of cardiac Troponin I (Supplemental Figure VIIC and VIID). Additionally, SIRT3 activation levels were not altered by the combination treatment with treprostinil and metformin in the RV (Supplemental Figure VIIC). Together, these data suggest that LKB1/SIRT3-indipendent activation of AMPK in the RV by treprostinil and metformin may be one of the mechanisms associated with improved cardiac function in metabolic syndrome-associated PH-HFpEF.

Discussion

Using high-fat feeding, which induces mild metabolic syndrome-associated PH-HFpEF, we demonstrate that chronic treprostinil treatment reduces hyperglycemia, glucose intolerance and pulmonary hypertension. We also demonstrate that treprostinil has a beneficial effect on glucose homeostasis, with comparable efficacy to metformin alone in rats with more profound metabolic syndrome-associated PH-HFpEF. Additionally, our data show that early chronic supplementation of treprostinil lowers pulmonary pressures and that a late treatment with both treprostinil and metformin normalizes hyperglycemia and improves cardiac function by a mechanism involving, at least in part, AMPK activation in skeletal muscle and the RV, along with improved insulin resistance. These findings are important as both cardiopulmonary and metabolic defects in metabolic syndrome-associated PH-HFpEF were highly modifiable in our study by treprostinil, a US Food and Drug Administration-approved drug for PAH, and metformin, which is the most commonly prescribed drug for the treatment of type 2 diabetes, demonstrating the potential for repurposing these drugs for the early treatment of PH-HFpEF.

To date, no approved specific medication or consensus therapeutic strategy for PH-HFpEF is available. Targeting left-sided filling pressure with PA pressure monitoring device to adjust doses of diuretic therapy has been shown to reduce HF hospitalization, but no changes with respect to PA pressure was observed in HFpEF patients.[39] Additionally, the use of the current approved treatments for PAH, such as sildenafil, riociguat, bosentan and macitentan, which act on nitric oxide or endothelin-1 pathways, is controversial and has been shown to be ineffective or even harmful in patients with PH-HFpEF.[10–14, 40] While the search for effective therapies for PH-HFpEF remains challenging, a significant breakthrough in cardiology represented by a recent finding that some antidiabetic drugs, such as metformin and sodium glucose co-transporter 2 inhibitors, are associated with reduction in HF hospitalizations and lower mortality.[41–43] These exciting findings have made metabolic syndrome-targeting therapies a potential strategy for treating heart failure, with several ongoing clinical trials evaluating the effects of SGLT2 inhibitors in HFpEF patients taking place at present (NCT03057951, NCT03619213 and NCT03030235).

In addition to HFpEF, several new discoveries over the past decade have revealed a ~35% prevalence of metabolic syndrome in PAH patients[4]. It has also been shown that the prevalence of insulin resistance is higher among female PAH patients and is associated with reduced survival compared to the insulin sensitive counterparts.[44] Moreover, coexistence of diabetes is associated with reduced 10-year survival in PAH patients compared to those without diabetes.[45] Targeting metabolic syndrome with rosiglitazone, an antidiabetic drug, as well as a peroxisome proliferator-activated receptor-gamma activator, has been shown to increase plasma adiponectin, reduce hyperglycemia and reverse increased RVSP and RV hypertrophy in HFD-treated apolipoprotein E mice.[29] Additionally, preventative treatment with metformin in HFD-treated C57/B16 mice has been shown to improve insulin resistance, reduce lipid accumulation and decrease right ventricular systolic pressure.[16] These data further support the idea that targeting metabolic syndrome may be a viable strategy for the treatment of metabolic syndrome-associated PAH and have led to initiation of a clinical trial designed to evaluate the effect of metformin in PAH patients (NCT03617458).

In contrast to patients with HFpEF or PAH, whose prevalence of metabolic syndrome is approximately 35%, clinical observations have shown that patients with PH-HFpEF have higher prevalence of hypertension, obesity, diabetes and hyperlipidemia. According to Robbins et al, more than 90% of PH-HFpEF patients have been shown to have two or more features of metabolic syndrome.[4] Consistent with these findings, more than 90% comorbidities of hypertension, diabetes and hyperlipidemia with a high body mass index value of 42 were observed in a patient cohort of PH-HFpEF recruited by Simon et al for evaluating the effect of nitrite,[46] a drug which has been shown to improve metabolic syndrome and cardiopulmonary hemodynamics in the Ob-Su rat model of PH-HFpEF with a similar effect to metformin.[15] Most recently, Ranchoux et al developed a new pre-clinical model of metabolic syndrome-associated PH-HFpEF using SAB with a combination treatment of olanzapine, an antidepressant known to induce the adverse effect of metabolic syndrome, and HFD.[17] This model confirms the idea that metabolic syndrome exacerbates Group 2 PH. Using this model, metformin treatment significantly reversed plasma leptin, visceral fat and elevated pulmonary pressure, further supporting the therapeutic potential of metabolic syndrome-targeting strategy for the treatment of PH-HFpEF.[17] While the effects of the first-line and second-line drugs for metabolic syndrome, metformin and SGLT2 inhibitor empagliflozin, respectively, are currently under investigation in clinical trials which include PH-HFpEF or HF patients with diabetes (NCT03629340 and NCT03030222), our new findings provide additional evidence that improved metabolic syndrome and insulin resistance may be a strategy for the early treatment of metabolic syndrome-associated PH-HFpEF. Our observations may open a new avenue for an early combination therapy of treprostinil and metformin in the management of PH-HFpEF in future studies.

Several studies have suggested that microvascular endothelial dysfunction caused by systemic inflammation and oxidative stress is the likely driving force that links insulin resistance to the development of metabolic syndrome-associated PH-HFpEF.[47–49] This may be related to nitric oxide deficiency and the subsequent impairment of cyclin guanosine monophosphate/protein kinase G signaling in cardiomyocytes, which results in LV stiffness and abnormal relaxation, left atrial impairment and ultimately an increase in pulmonary artery pressures.[50, 51] As endothelial dysfunction contributes to insulin resistance, impaired

insulin action has been shown to reverberate a negative feedback loop to worsen endothelial dysfunction and further disturb glucose and lipid metabolism, which in turn trigger inflammation, induce oxidative stress and exacerbate endothelial dysfunction and insulin resistance. In fact, lipid accumulation in skeletal muscle has been shown to create a pro-inflammatory state and promote insulin resistance in mice with bone morphogenetic protein receptor 2 mutation, the most commonly identified genetic mutation in PAH, as well as in patients with HFpEF.[52–54] Lipid accumulation in the RV has also been linked to the development of PH.[16] Besides, hyperglycemia-induced overproduction of mitochondrial reactive oxygen species, elevated oxidative stress and mitochondrial dysfunction have been suggested as major factors in the pathogenesis of diabetic complications.[55] Impaired mitochondrial function and elevated oxidative stress by persistent hyperglycemia in cardiomyocytes has been associated with LV diastolic dysfunction and disease worsening in HFpEF.[56] While the impact of hyperglycemia on the stabilization of hypoxia-inducible factor 1α is still unclear, hyperglycemia-induced cellular hypoxia has been shown to promote hyperglycemic complications via induction of endothelin-1 and fibronection in endothelial cells.[57] Hyperglycemia also regulates PASMCs proliferation and migration through increased expression of Smad ubiquitin ligase (Smurf-1) to down-regulate BMP signaling in PAH.[58] Moreover, skeletal muscle glucose intolerance, mitochondrial dysfunction and metabolism defects have also been closely associated with exercise intolerance and worsened functional capacity in patients with either PAH or HFpEF.[59–61]

We are intrigued by the chronically sustainable effect of treprostinil in improving glucose homeostasis, including hyperglycemia and glucose intolerance, with a comparable efficacy to that of metformin alone in rats with more profound diabetes associated with dyslipidemia and hypertension. While a prostacyclin derivative, beraprost sodium, has been recently shown to suppress the development of diabetes by improving glucose intolerance and insulin resistance in obese Zucker fatty rats, the mechanism behind this observation has not been established.[19] Here, we show that the glucose-lowering effect of treprostinil seems to be mediated predominantly by insulin-independent skeletal muscle glucose uptake through increased muscle LKB1/SIR3-AMPK-GLUT4 activation. Consistent with recent findings that activation of skeletal muscle AMPK by pan-AMPK activators is capable of promoting insulin-independent glucose lowering and uptake in diabetic mice and monkeys without affecting hepatic glucose production,[62, 63] our data suggest that skeletal muscle AMPK activation by treprostinil may be crucial in the regulation of antihyperglycemic action. Yet, whether this skeletal muscle AMPK activation-mediated glucose lowering effect can be extended to all prostanoids and/or to various routes of drug administration (e.g. inhalation and oral), needs to be further investigated. Based on the US Food and Drug Administration report, among 33,568 people who have adverse effects when taking treprostinil, 0.1% have hypoglycemia, particularly to those who are female, age 60 or older, and also take bosentan. While the mechanism related to this observation is unknown, one of the metabolites of treprostinil, the glucuronoconjugate of treprostinil, has been shown to increase glucose consumption during metabolism.[64, 65] In the present study, our results show that plasma from treprosinil-treated animals, rather than direct addition of treprostinil, induces LKB1/SIRT3-AMPK activation *in vitro*. Whether or not the glucose lowering effect of treprostinil is dependent on the glucuronidated metabolites needs to be further evaluated.

Our data also unexpectedly reveal that treprostinil synergizes with metformin in improving abnormal glucose regulation to normal glucose tolerance via skeletal muscle LKB1/SIR3-AMPK-mediated glucose uptake and improved insulin resistance. These phenomena are associated with improved TAPSE and PAAT/ET in more severely affected SU5416/obese ZSF1 rats. Similar to SIRT3, the mitochondrial deacetylase that has been shown to modulate diabetes through the maintenance of skeletal muscle insulin action, glucose disposal and mitochondrial function,[66] the tumor suppressor LKB1 is involved in the regulation of insulin sensitivity and glucose homeostasis.[67] LKB1 has also been shown to regulate lipid oxidation independently of AMPK during exercise.[68] Given that treprostinil improves lipemia without affecting triglycerides and total cholesterol, future experiments are needed to determine whether LKB1-mediated lipid oxidation is involved in this context. Along with improved hyperglycemia and pulmonary pressures by nitrite and metformin alone via skeletal muscle SIRT3-AMPK activation,[15] our data suggest a common mechanism contributed, at least in part, by skeletal muscle LKB1/SIRT3-AMPK signaling to the early treatment of metabolic syndrome-associated PH-HFpEF. However, the relative contributions to improved cardiac function and/or pulmonary pressure observed with these drugs by skeletal muscle-mediated improvement of glucose metabolism and lung signaling remain uncertain.

In addition to skeletal muscle AMPK activation, our data show a connection between RV AMPK activation and improved RV function in Ob-Su rats treated with treprostinil and metformin. AMPK activation has been shown to inhibit cardiomyocyte hypertrophy via suppression of protein synthesis and gene transcription,[69, 70] however, RV mass/hypertrophy was not affected by the combination treatment with treprostinil and metformin in this study. AMPK has also been shown to play a role in regulating cardiac function and cardiomyocyte contractility through phosphorylating cardiac Troponin I at Ser150.[35–37] We found that treprostinil and metformin-mediated RV AMPK activation is not associated with phosphorylation levels of cardiac Troponin I, suggesting the beneficial effect of treprostinil and metformin on the RV is independent of improved contractile function. Our data indicate that late treatment with treprostinil alone does not alter RV hypertrophy, TAPSE and phosphorylation levels of AMPK and Troponin I in the RV of Ob-Su rats. This observation is consistent with previous findings that treprostinil treatment has no significant effect on cardiomyocyte contractility, RV hypertrophy and RV function in rat cardiomyocytes or in pulmonary trunk banding model of pressure overload-induced right ventricular hypertrophy and failure.[71, 72] Recently, metformin has been shown to prevent RV dysfunction and myocardial steatosis via improved insulin resistance and reduced RV lipid accumulation in HFD-treated C57/B16 mice.[16] Infusion of epoprostenol has also been shown to reduce RV size, decrease PA resistance and improve RV function via its vasodilating effect in patients with PAH and in dogs with load-induced acute RV failure.[73, 74] Further studies are needed to determine whether the reduction of RV lipid content together with pulmonary and/or systemic vasodilation are the mechanisms through which treprostinil and metformin may exert their beneficial effects.

Although not conclusive, our data suggest a potential role of treprostinil as an early treatment for mild metabolic syndrome-associated PH-HFpEF. Our data also show that treprostinil has similar effects to metformin, improving insulin-independent glucose lowering and disposal by activating LKB1/SIRT3-AMPK-GLUT4 in skeletal muscle. In

addition, we show that early chronic supplementation of treprostinil lowers pulmonary pressures and that a late combination therapy of treprostinil and metformin normalizes hyperglycemia and improves cardiac function via skeletal muscle and RV AMPK activation in rats with more severe PH-HFpEF. As a whole, our study provides insights into the development of skeletal muscle LKB1/SIRT3-AMPK- and/or RV AMPK-targeted therapies and presents an early treatment strategy for a combination therapy of treprostinil and metformin for PH-HFpEF in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMPK	AMP-activated protein kinase
CaMKK2	calcium/calmodulin-dependent protein kinase kinase 2
GLUT4	glucose transporter 4
HbA1c	hemoglobin A1c
HFD	high-fat diet
HFpEF	heart failure with preserved ejection fraction
LKB1	liver kinase B1
LV	left ventricle and or left ventricular
PAAT/ET	pulmonary artery acceleration time to ejection time ratio
PAH	pulmonary arterial hypertension
PH	pulmonary hypertension
PH-HFpEF	pulmonary hypertension associated with heart failure with preserved ejection fraction
RV	right ventricle and/or right ventricular
SIRT3	sirtuin-3

TAK1	transforming growth factor-β-activated kinase-1
TAPSE	tricuspid annular plane systolic excursion

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Highlights

- Chronic treprostinil treatment lowers hyperglycemia, glucose intolerance and pulmonary pressures.
- Treprostinil has a beneficial effect on lowering glucose via activation of LKB1/SIRT3-AMPK-GLUT4 pathway in skeletal muscle with efficacy comparable to metformin.
- Late treatment with treprostinil and metformin normalizes hyperglycemia and improves cardiac function by AMPK activation in skeletal muscle and the RV.
- This study provides insights into the development of skeletal muscle LKB1/SIRT3-AMPK- and/or RV AMPK-targeted therapies for metabolic syndrome-associated PH-HFpEF.

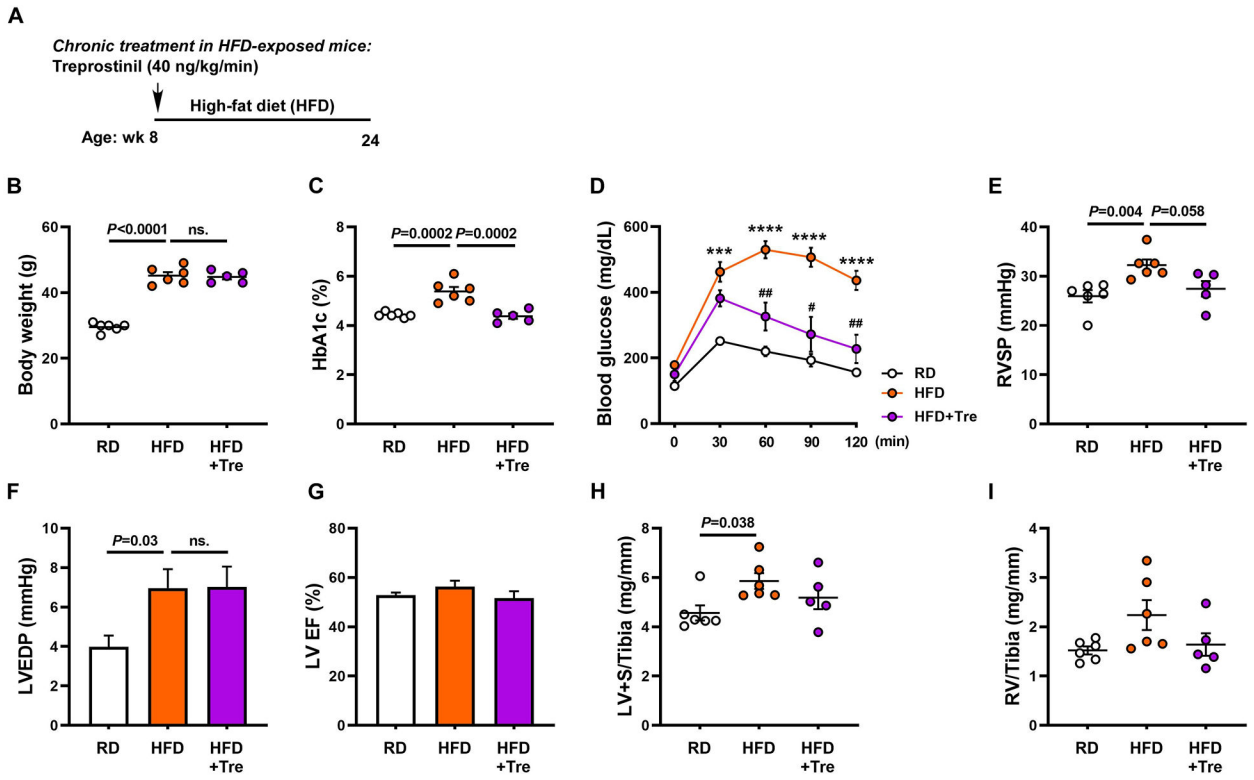

Figure 1. Chronic treprostinil treatment reduces hyperglycemia, glucose intolerance and pulmonary hypertension in mice with high-fat diet-induced PH-HFpEF.

(**A**) Treprostinil (40 ng/kg/min) was given through osmotic minipumps for 16 weeks in mice fed with a HFD (60% lipids/kcal). Body weights (**B**), HbA1c levels (**C**), glucose tolerant abilities (**D**), right ventricular systolic pressures (RVSP, **E**), left ventricular end diastolic pressure (LVEDP, **F**), left ventricular ejection fraction (LVEF, **G**) were measured. **H** and **I**, LV and RV mass normalized to tibial length. Data are mean ± SEM; n = 5–6 mice/group; For D, ***$P < 0.001$ and ****$P < 0.0001$ vs. RD; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. HFD.

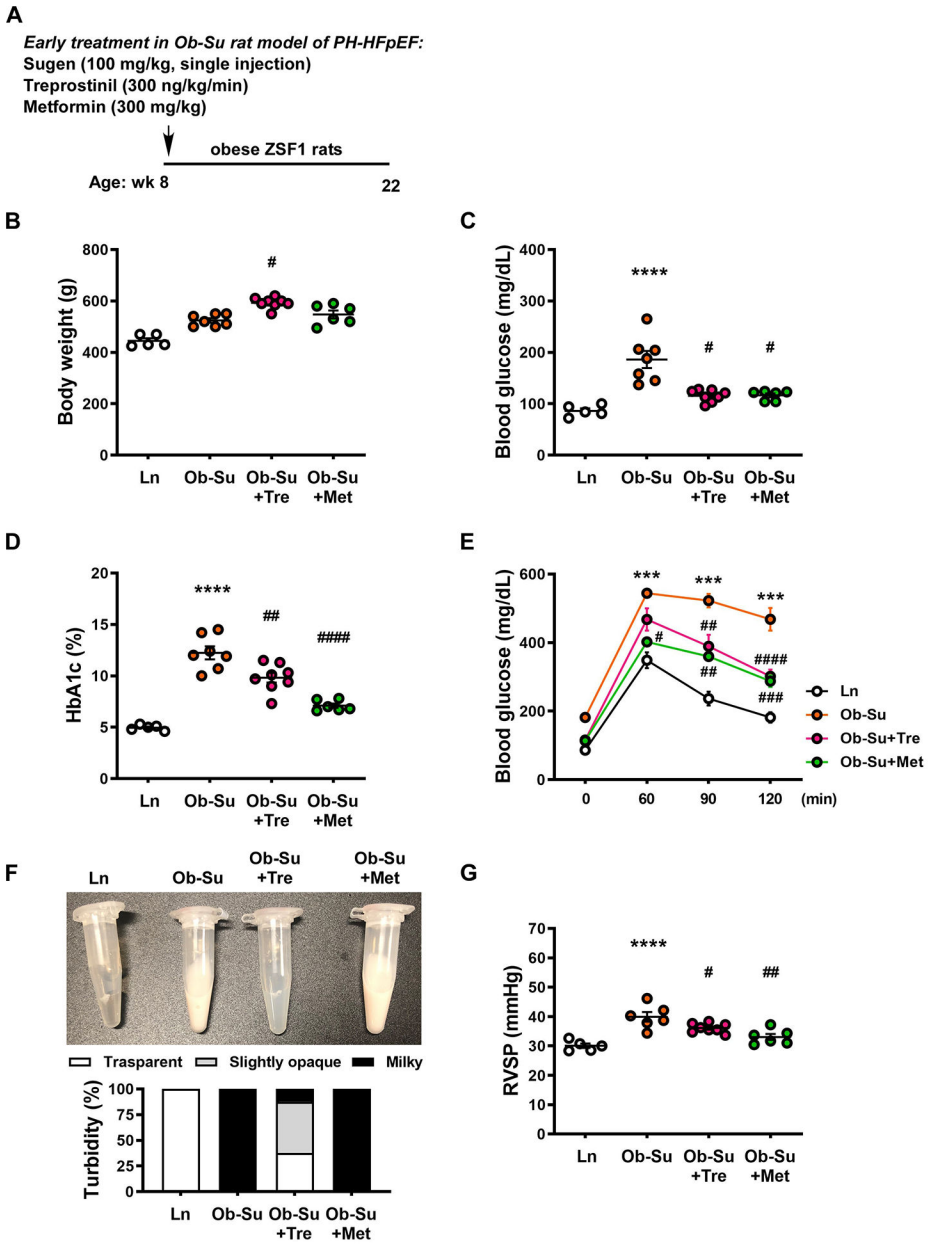

Figure 2. Early treatment with treprostinil improves hyperglycemia and lowers pulmonary pressures in SU5416/obese ZSF1 rats.

A, Treprostinil (300 ng/kg/min) and metformin (300 mg/kg) were given through osmotic minipumps and drinking water, respectively, for 14 weeks to 8-week old SU5416/obese ZSF1 rats (Ob-Su). Body weights (**B**), fasting blood glucose levels (**C**), HbA1c levels (**D**), glucose tolerant abilities (**E**) and right ventricular systolic pressures (RVSP, **G**) were measured. **F**, Representative images and percentage of milky, slightly opaque and transparent plasma samples in different groups. Data are mean ± SEM; $n = 5–8$ rats/group; $^{*}P < 0.05$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ vs. ln; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$ vs. Ob-Su.

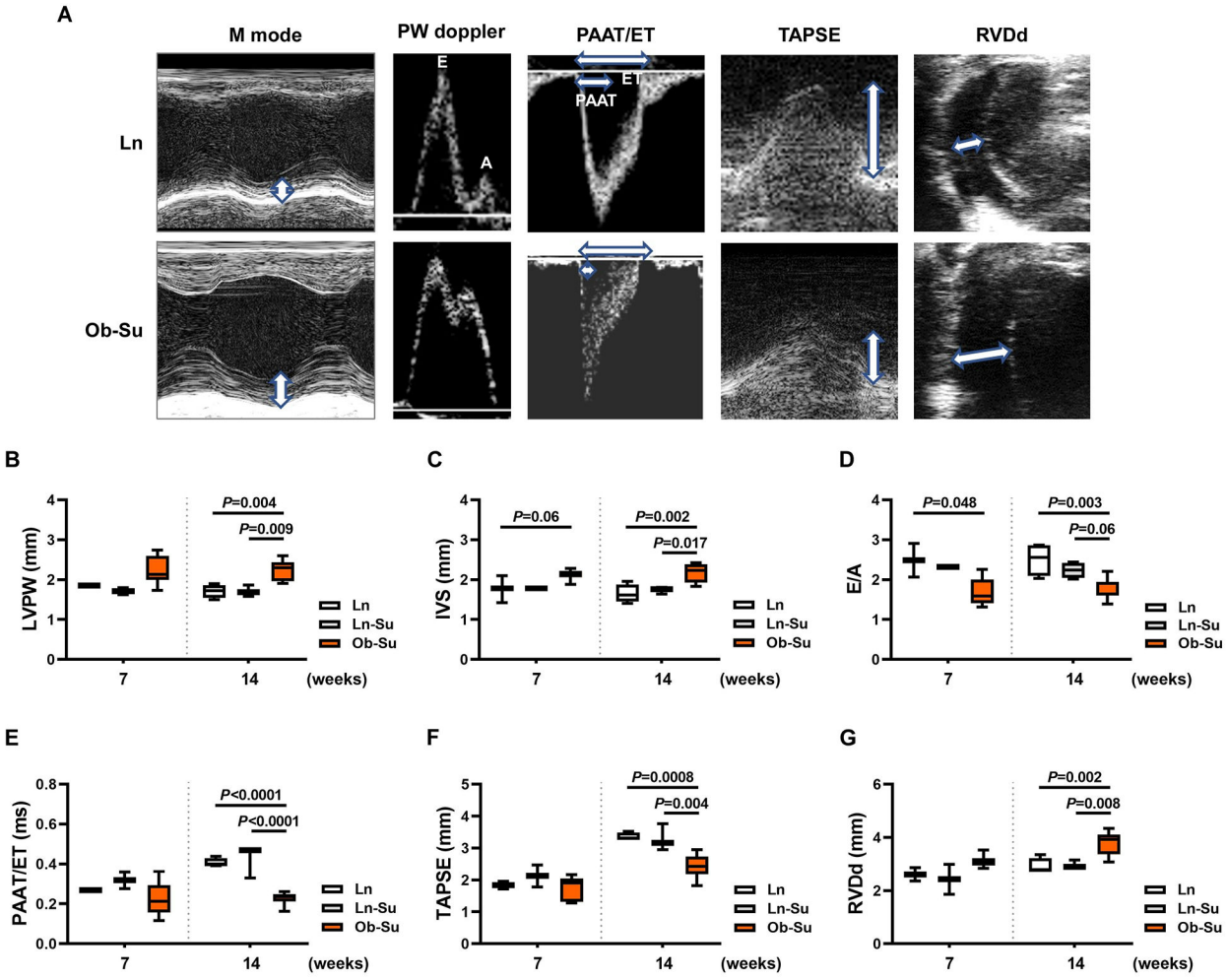

Figure 3. SU5416/obese ZSF1 rats progressively develop metabolic syndrome-associated PH-HFpEF.

(**A**) Representative echocardiographic images assessed after 14 weeks of SU5416 administration to obese ZSF1 rats (SU5416/obese ZSF1, labeled as Ob-Su). Seven and 14 weeks after SU5416 administration in obese ZSF1 rats, the end-diastolic thickness of left ventricular posterior wall (LVPW, **B**) and interventricular septum (IVS, **C**), the ratio of early diastolic mitral annular velocity to mitral peak velocity of late filling (E/A, **D**), the ratio of pulmonary artery acceleration time to ejection time (PAAT/ET, **E**), tricuspid annular plane systolic excursion (TAPSE, **F**) and right ventricular dimension at end-diastole (RVDd, **G**) were measured. Data are mean ± SEM; n = 3–8 rats/group.

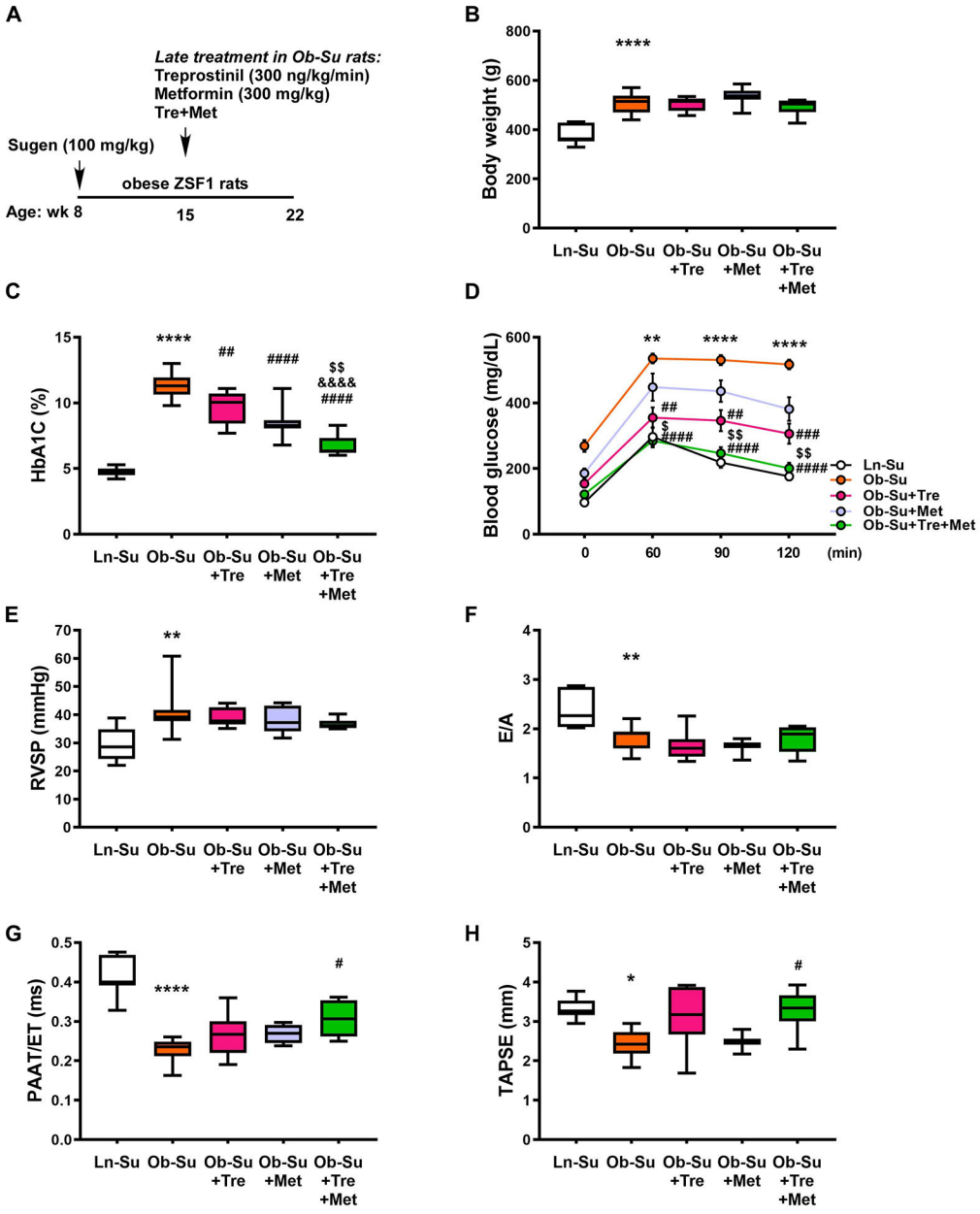

Figure 4. Treatment with treprostinil and metformin normalizes hyperglycemia and improves cardiac function in SU5416/obese ZSF1 rats.

A, Treprostinil (300 ng/kg/min, osmotic minipumps), alone or in combination with metformin (300 mg/kg, drinking water), was given 7 weeks after a single injection of SU5416 (100 mg/kg) to obese ZSF1 rats. Body weights (**B**), HbA1c levels (**C**), glucose tolerant abilities (**D**), right ventricular systolic pressures (RVSP, **E**) and the ratio of early diastolic mitral annular velocity to mitral peak velocity of late filling (E/A, **F**) were measured. The ratio of pulmonary artery acceleration time to ejection time (PAAT/ET, **G**) and tricuspid annular plane systolic excursion (TAPSE, **H**) were measured. Data are mean ± SEM; $n = 4–13$ rats/group; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$ vs. Ln-Su; $^{\#}P < 0.05$,

$^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$ vs. Ob-Su; $^{\&\&\&\&}P < 0.0001$ vs. Ob-Su+Tre; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ vs. Ob-Su+Met.

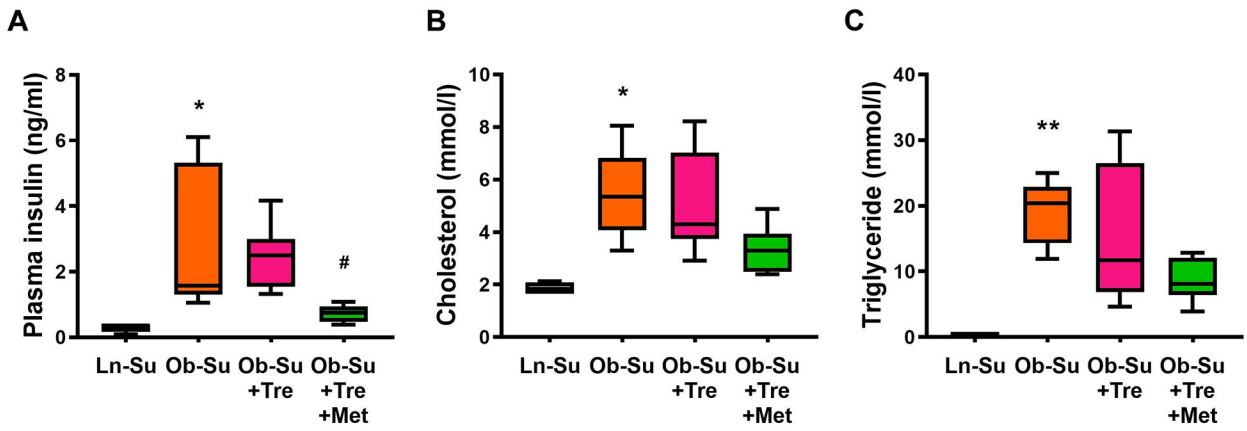

Figure 5. Treatment with treprostinil and metformin ameliorates hyperinsulinemia and hypercreatininemia.

Treprostinil (300 ng/kg/min, osmotic minipumps), alone or in combination with metformin (300 mg/kg, drinking water), was given 7 weeks after a single injection of SU5416 (100 mg/kg) to obese ZSF1 rats. Seven weeks after the treatment, plasma samples were collected and circulating levels of insulin (**A**), cholesterol (**B**) and triglyceride (**C**) were measured. Data are mean ± SEM; $n = 4–8$ rats/group; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Ln-Su; $^{\#}P < 0.05$ vs. Ob-Su.

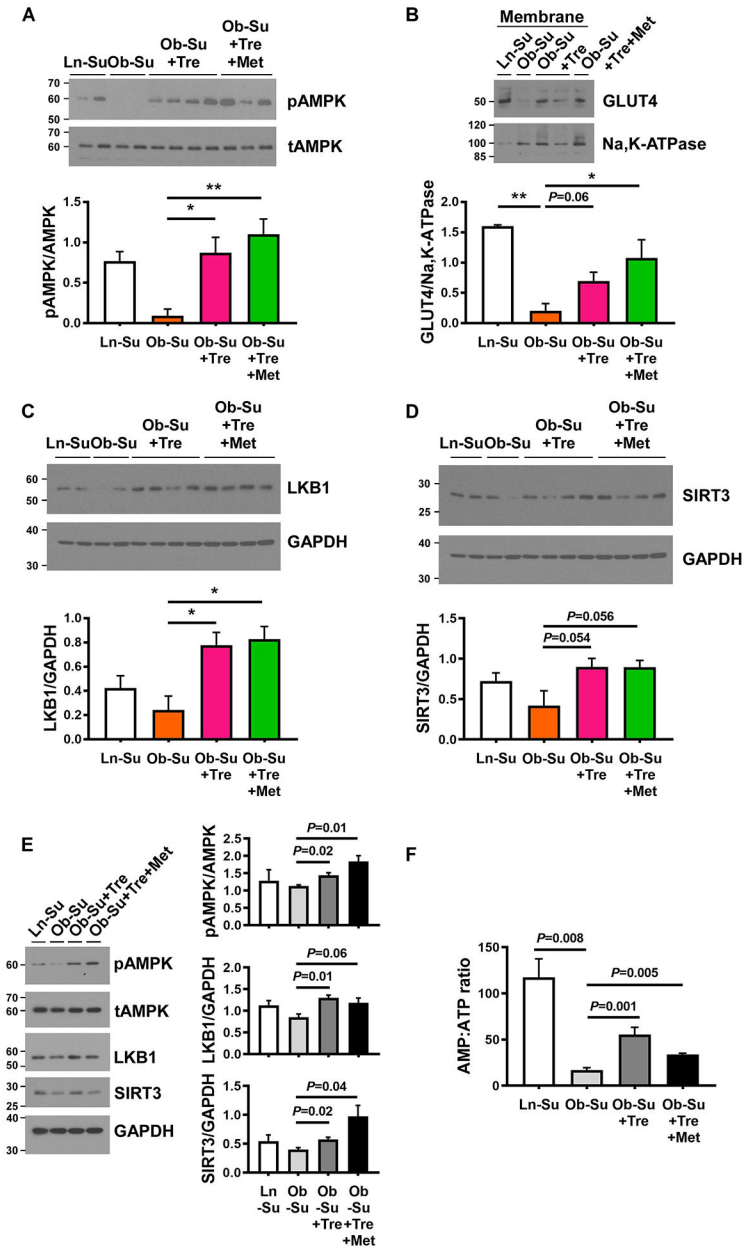

Figure 6. Treprostinil and metformin improves metabolic syndrome-associated PH-HFpEF via activation of LKB1/SIRT3-AMPK-GLUT4 signaling pathway.

A through D, Skeletal muscle samples were collected from SU5416 (100 mg/kg) treated lean and obese ZSF1 rats (labeled as Ln-Su and Ob-Su, respectively), in the presence or absence of treprostinil (300 ng/kg/min, osmotic minipumps) alone or in combination with metformin (300 mg/kg, drinking water). Effect of treprostinil on AMPK-stimulated glucose uptake was detected by Western blot analyses. **B**, Representative Western blots for GLUT4 expression in membrane protein extracts from skeletal muscle. Representative Western blots for LKB1 expression (**C**) and SIRT3 activation (**D**) levels in skeletal muscle. **E and F**, C2C12 skeletal muscle myoblasts were treated with plasma obtained from Ln-Su rats or Ob-Su rats, treated with or without treprostinil, alone or in combination with metformin, for 48

hours at the end of the differentiation period. AMPK phosphorylation, LKB1 expression and SIRT3 activation levels (**E**), as well as the AMP:ATP ratio (**F**) were measured. Data are mean ± SEM; $n = 3–6$ (*in-vivo*) or 3 (*in-vitro*); $*P < 0.05$ and $**P < 0.01$.

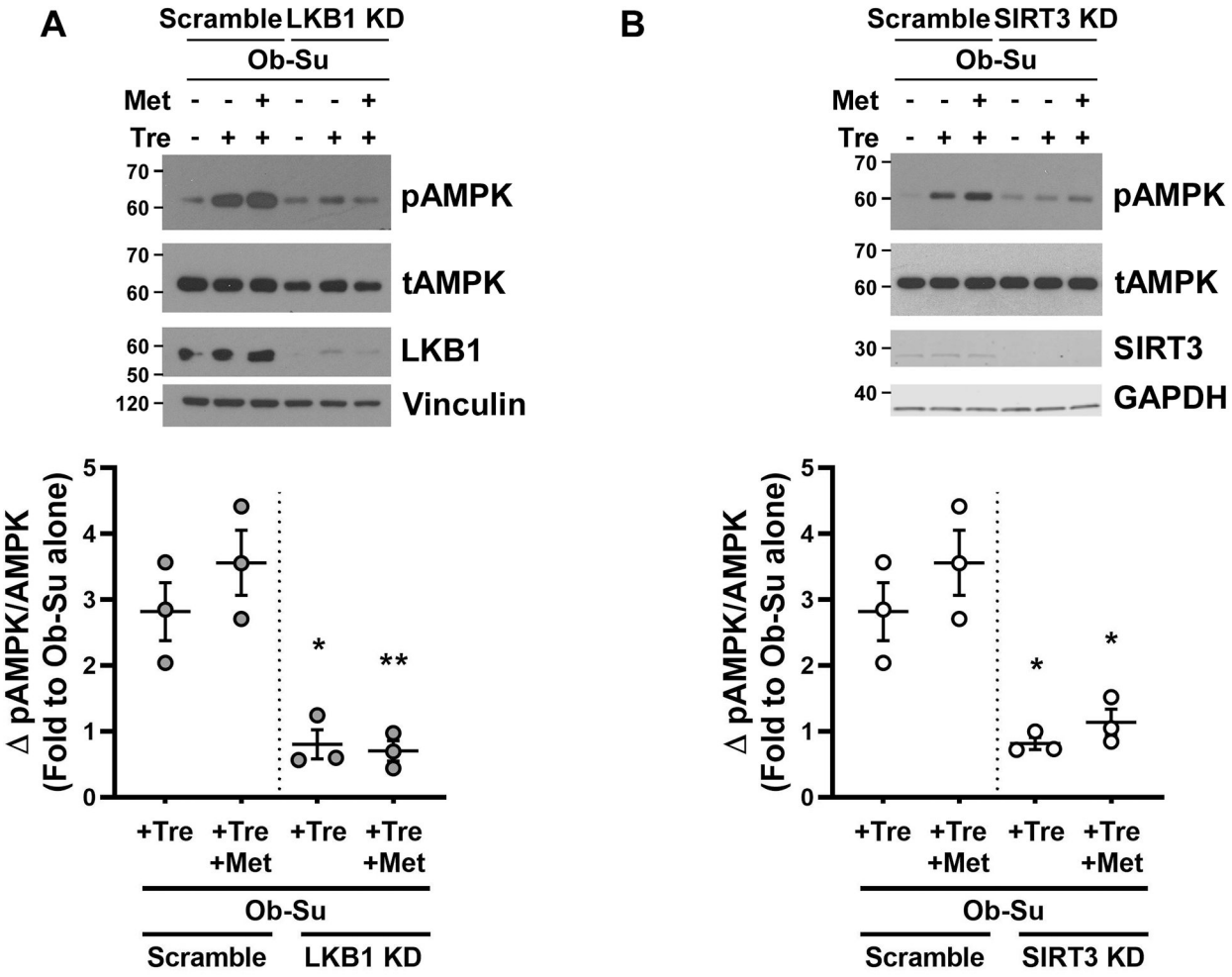

Figure 7. LKB1 and SIRT3 are required for treprostinil-mediated AMPK activation in C2C12 skeletal muscle cells.

C2C12 skeletal muscle cells were transiently transfected with siRNA targeting LKB1, SIRT3 or scrambled control 24 hours before stimulation with plasma obtained from treprostinil-treated Ob-Su rats for an additional 48 hours at the end of the differentiation period. Effect of LKB1 or SIRT3 knockdown (KD) on treprostinil-mediated AMPK activation was measured by Western blots. Dot plots show the fold change of AMPK phosphorylation relative to stimulation with plasma obtained from Ob-Su rats. Data are mean ± SEM; $n = 3$; *$P < 0.05$ and **$P < 0.01$ compared to correlated scrambled control groups.

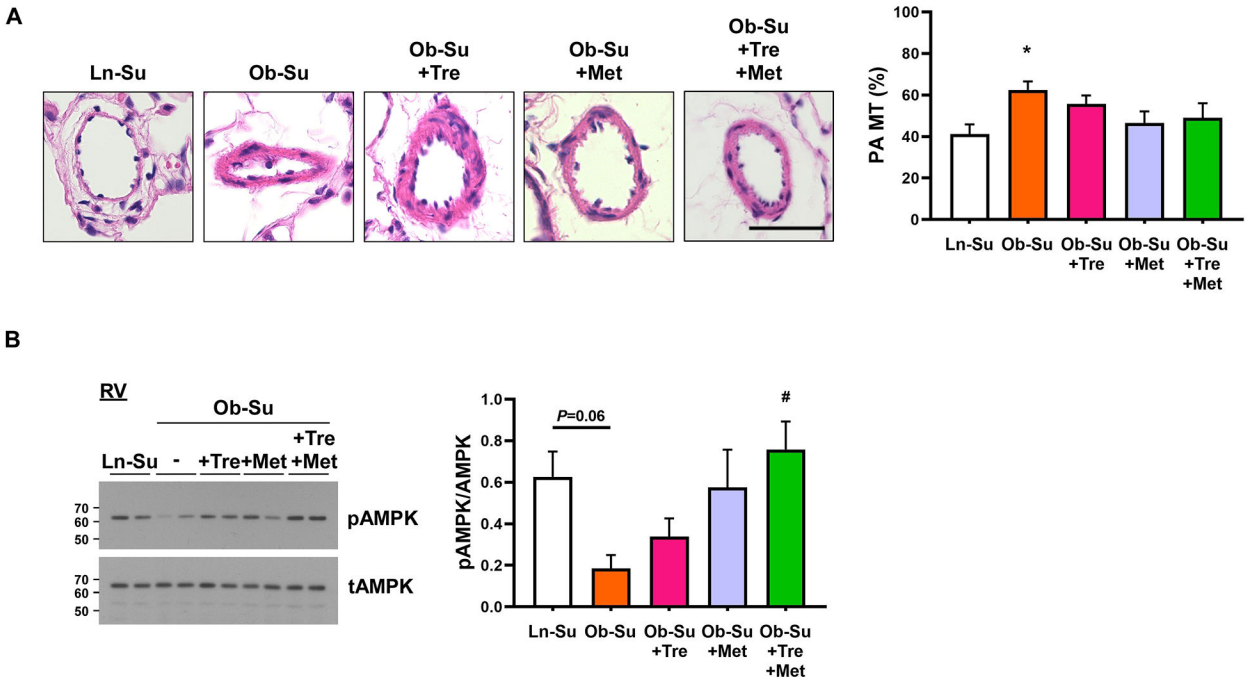

Figure 8. Treatment with treprostinil and metformin activates AMPK in the RV.

Treprostinil (300 ng/kg/min, osmotic minipumps), alone or in combination with metformin (300 mg/kg, drinking water), was given 7 weeks after a single injection of SU5416 (100 mg/kg) to obese ZSF1 rats. **A**, Seven weeks after the treatment, lung tissue sections were subjected to pulmonary artery (PA) medial wall thickness (MT) analyses. Scale bar = 50 μm. 40X. **B**, Effects of treprostinil and/or metformin on AMPK activation in the RV were detected by Western blot analyses. Data are mean ± SEM; n = 3–5 rats/group; $^{*}P < 0.05$ vs. Ln-Su and $^{\#}P < 0.05$ vs. Ob-Su.