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## SIRT3-AMPK Activation by Nitrite and Metformin Improves Hyperglycemia and Normalizes Pulmonary Hypertension Associated with Heart Failure with Preserved Ejection Fraction (PH-HFpEF)

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### Abstract

**Background**—Pulmonary hypertension associated with heart failure with preserved ejection fraction (PH-HFpEF) is an increasingly recognized clinical complication of metabolic syndrome. No adequate animal model of PH-HFpEF is available and no effective therapies have been identified to date. A recent study suggested that dietary nitrate improves insulin resistance in eNOS null mice, and multiple studies have reported that both nitrate and its active metabolite, nitrite, have therapeutic activity in pre-clinical models of PH.

**Methods and Results**—In order to evaluate the efficacy and mechanism of nitrite in metabolic syndrome associated with PH-HFpEF, we developed a “two-hit” PH-HFpEF model in rats with multiple features of metabolic syndrome due to double leptin receptor defect (obese ZSF1) with the combined treatment of VEGF receptor blocker SU5416. Chronic oral nitrite treatment improved hyperglycemia in obese ZSF1 rats by a process that requires skeletal muscle SIRT3-

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AMPK-GLUT4 signaling. The glucose lowering effect of nitrite was abolished in SIRT3 deficient human skeletal muscle cells, as well as in SIRT3 knockout mice fed a high-fat diet. Skeletal muscle biopsies from humans with metabolic syndrome after 12 weeks of oral sodium nitrite and nitrate treatment (IND#115926) displayed increased activation of SIRT3 and AMPK. Finally, early treatments with nitrite and metformin at the time of SU5416 injection reduced pulmonary pressures and vascular remodeling in the PH-HFpEF model with robust activation of skeletal muscle SIRT3 and AMPK.

**Conclusions**—These studies validate a rodent model of metabolic syndrome and PH-HFpEF, suggesting a potential role of nitrite and metformin as a preventative treatment for this disease.

## Keywords

PH-HFpEF; SIRT3; AMPK; metabolic syndrome; hypertension; pulmonary

## Introduction

Group 2 pulmonary hypertension (PH) or post-capillary pulmonary venous hypertension is known to occur secondary to left ventricular (LV) systolic or diastolic dysfunction. The latter is more commonly referred to as heart failure with preserved ejection fraction (HFpEF) or nonsystolic heart failure, and is the most common cause of Group 2 PH. With chronic elevations in the diastolic filling pressure of the left heart, the pulmonary vasculature can vasoconstrict and undergo pathological remodeling, leading to an elevated pulmonary vascular resistance (PVR), high transpulmonary pressure gradients, and secondary right ventricular (RV) hypertrophy and dilation<sup>1–4</sup>. Therefore, PH-HFpEF patients develop more severe symptoms than HFpEF patients and suffer significant exercise intolerance, frequent hospitalization, and reduced survival<sup>3</sup>. Since PH-HFpEF patients usually have a higher prevalence of cardiovascular comorbidities including hypertension, diabetes, obesity, and coronary artery disease than patients with pulmonary arterial hypertension (PAH), PH-HFpEF is increasingly recognized as a clinical complication of metabolic syndrome<sup>3–5</sup>. In fact, a recent observational study has shown that the development of PH-HFpEF is commonly associated with two or more features of metabolic syndrome<sup>6</sup>. To date, no animal models of PH-HFpEF associated with metabolic syndrome have been established and no specific therapy has been identified.

Reduced bioavailability and impaired production of nitric oxide (NO) are thought to contribute to the development of both metabolic syndrome and PH<sup>7–12</sup>. An increasing number of therapeutic approaches, which enhance NO generation and bioactivity, have been proposed over the past decade and include administration of nitrite and nitrate<sup>13, 14</sup>. Recent publications from our group and others have shown that nitrite exhibits therapeutic efficacy in mouse, rat, and sheep pre-clinical models of PH<sup>15–17</sup>, and also appears to be well tolerated in humans<sup>18</sup>. Additionally, oral supplementation of nitrate prevents the development of hypoxia- and monocrotaline-induced PH<sup>19</sup>. Furthermore, a recent study suggests that dietary supplementation of nitrate reverses features of metabolic syndrome in eNOS deficient mice<sup>20</sup>, yet the mechanism behind this observation and the role of its active metabolite, nitrite, on metabolic syndrome and metabolic syndrome-associated PH-HFpEF remains elusive.

AMP-activated protein kinase (AMPK) is a cellular energy sensor that participates in many metabolic processes and controls liver and skeletal muscle glucose metabolism<sup>21</sup>. Dysregulation of AMPK and its signaling network have been associated with metabolic syndrome, heart failure, and PH<sup>22–27</sup>. Several studies have shown that NO donors increase glucose uptake and GLUT4 expression via AMPK activation<sup>28, 29</sup> and nitrite has been shown to increase AMPK activation in cardiomyocytes, identifying AMPK as a candidate signaling modulator for the effects of nitrate and nitrite on glucose homeostasis<sup>30</sup>. Activation of AMPK can be mediated by the upstream kinases, including liver kinase B1 (LKB1), calcium/calmodulin-dependent kinases (CaMKK and CaMKII), and TGF $\beta$ -activated kinase-1 (TAK1)<sup>31–34</sup>. Recently, sirtuin-3 (SIRT3), a major mitochondrial deacetylase that is upregulated with diet and exercise, has also been shown to activate AMPK in skeletal muscle and neurons<sup>35–37</sup>. On the basis of such observations, we hypothesized that nitrite might improve metabolic syndrome and metabolic syndrome-associated PH-HFpEF via canonical insulin-dependent or non-canonical AMPK-dependent signaling pathways.

In addition to nitrite, metformin, the canonical AMPK activator and the first-line drug for modulating metabolic syndrome, has also been shown to prevent the development of hypoxia- and monocrotaline-induced PH<sup>38</sup>. Hence, in the present study, we sought to evaluate the effectiveness and mechanism of nitrite and metformin in the treatment of metabolic syndrome-associated PH-HFpEF.

## Methods

Methods are expended in Online Data Supplement.

### Animal studies

All experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Eight-week and twenty-three-week old male obese ZSF1 rats, and their lean littermates (Charles River, Wilmington, MA) were used in this study. Sodium nitrite (Sigma) and metformin (Spectrum) were given in drinking water. VEGFR2 antagonist SU5416 (Cauldron Chemicals, East Lynne, R.S.A; kindly provided as unrestricted gift by Dr. Claude Piche) was dissolved in CMC buffer (0.5 % sodium carboxymethyl cellulose, 0.4 % polysorbate 80, 0.9 % sodium chloride, and 0.9 % benzyl alcohol) and was given as a single 100 mg/kg subcutaneous injection to lean and obese ZSF1 rats. Eight-week old male 129-SIRT3 knockout (KO) mice (The Jackson Laboratory, Bar Harbor, ME) were fed a high-fat diet (HFD, 60% fat in kcal, Research diet) for 20 weeks. All animals were maintained in a normoxic environment.

### Hemodynamic and ventricular measurements

Briefly, rats were anesthetized with isoflurane (1–2%). The trachea was cannulated, and rats were ventilated at the rate of 75–80 BPM with a tidal volume of 2.5 ml. Right ventricular systolic pressure, left ventricular end diastolic pressure, left ventricular ejection fraction, and mean arterial blood pressure were measured using admittance pressure-volume catheter. Right and left ventricular weights normalized by tibial length were used as indexes of

ventricular mass. Fulton index (weight of RV/weight of LV+septum) was used as an index of RV hypertrophy.

### Human skeletal muscle cell culture

Myoblasts isolated from biopsied vastus lateralis muscle obtained from lean and obese volunteers (BMI ~33 kg/m<sup>2</sup>)<sup>39</sup> were cultured in Hams F10 media (Invitrogen, Grand Island, NY) with 20% FBS, 1% penicillin/streptomycin (P/S), and fibroblastic growth factors (Invitrogen, Grand Island, NY). Passages 3–5 were used. Differentiation was induced using DMEM containing 1 g/L glucose, 110 mg/L sodium pyruvate, 2% FBS, and 1% P/S. Cells were allowed to differentiate for 7 days into mature myotubes. Medium was changed every 48 hours, and cells were incubated in a 5% CO<sub>2</sub>, 37 °C humidified atmosphere.

### Human subject recruitment and nitrite/nitrate supplementation

Human subjects with metabolic syndrome were recruited from university/community advertisements, the University of Pittsburgh Research Participant Registry and the ClinicalTrials.gov website (NCT01681810) to receive both sodium nitrite (20 mg, twice daily) and sodium nitrate (1000 mg, once daily), designed to maintain steady levels of nitrite in the circulation. The half-life in circulation following a single oral dose of nitrite is approximately 30–60 minutes<sup>40</sup>, and 6 hours for nitrate, which is then further reduced to nitrite by the oral mouth microbiome, maintaining elevated levels of nitrite in plasma for up to 24 hours<sup>41</sup>. The investigation was approved by the Institutional Review Board and informed consent was obtained from all participants before participation in accordance with the ethical guidelines of the University of Pittsburgh.

### Statistical analysis

Statistical analyses were performed using STATA 14.0 software (StatCorp., College Station, TX). Statistical significance between two independent groups was assessed by Mann-Whitney U test. Wilcoxon signed-rank test was used for comparing two paired groups. Statistical comparison among 3 groups was assessed by Kruskal-Wallis test followed by Dunn's *post-hoc* test with Bonferroni corrections for multiple comparisons. Mixed effect model with bootstrapping was used to compare 3 groups across time points. Values of  $P < 0.05$  were considered to be statistically significant.

## Results

### Development of a “two-hit” model of PH-HFpEF

To develop an animal model that can closely recapitulate the pathogenesis and clinical outcomes of human PH-HFpEF, we injected SU5416 (Sugen), VEGF receptor blocker that induces lung endothelial injury and/or apoptosis<sup>42</sup>, into rats with multiple features of metabolic syndrome, diabetic nephropathy, and diastolic dysfunction (obese ZSF1 rats, Figure 1A)<sup>43–45</sup>. Fourteen weeks after SU5416 administration, we found that SU5416-exposed obese ZSF1 rats (SU5416/ZSF1, labeled as Ob-Su) developed significantly higher right ventricular systolic pressure (RVSP,  $38.2 \pm 1.2$  mmHg;  $P = 0.0003$ ) compared to lean rats (Figure 1B). Concomitant with elevated right ventricular systolic pressure, the SU5416/ZSF1 rats exhibited higher left ventricular end diastolic pressure (LVEDP), preserved left

ventricular ejection fraction (LVEF), and increased mean right arterial pressures (mRAP) and mean arterial blood pressure (MABP) compared to lean rats (Figure 1C–1F). Elevated PVR and pulmonary vascular proliferative remodeling were also observed in the SU5416/ZSF1 rats (Figure 1G and 1H). Additionally, in comparison to lean rats, SU5416/ZSF1 rats had both RV and LV hypertrophy and increased fulton index (Figure 1I–K). All of these observed hemodynamic features in SU5416/ZSF1 rats are similar to the clinical characteristics of human PH-HFpEF<sup>4</sup>.

### **Chronic oral nitrite supplementation improves hyperglycemia and glucose intolerance in obese ZSF1 rats**

To assess the effect of chronic oral nitrite treatment on metabolic syndrome, two doses of nitrite (50 and 100 mg/L) were provided in drinking water for 14 weeks in obese ZSF1 rats (Figure 2A). There was no significant effect of nitrite on the body weights during this 14-week period (Figure 2B). Obese ZSF1 rats had higher plasma triglyceride levels than lean rats; however, triglyceride levels were not significantly different in nitrite-treated ZSF1 rats compared to untreated obese animals (Figure S1A). In contrast, fasting blood glucose and glycosylated hemoglobin (HbA1c) levels were lower in nitrite-treated obese ZSF1 rats compared to untreated obese animals (Figure 2C and 2D). Furthermore, as shown in Figure 2E, obese ZSF1 rats had impaired ability to maintain normal glucose levels during the oral glucose tolerance test, and chronic nitrite supplementation improved glucose intolerance in obese ZSF1 rats. Collectively, these data demonstrate that prolonged oral nitrite treatment improves hyperglycemia and glucose intolerance in obese ZSF1 rats independent of changes in body weight. These observations are similar to those observed with chronic nitrate therapy in the eNOS knockout mice<sup>20</sup>, suggesting that the bioconversion of nitrate to nitrite accounted for these observed effects.

### **Prolonged oral nitrite treatment improves hyperglycemia via activation of AMPK-GLUT4-mediated glucose uptake in obese ZSF1 rats**

To determine whether the beneficial effect of nitrite on reducing blood glucose and improving glucose intolerance in obese ZSF1 rats are related to changes in insulin secretion and  $\beta$ -cell homeostasis, we measured plasma insulin levels and quantified  $\beta$ -cell mass in these rats. A significant increase in plasma insulin concentration (Figure 3A) and the mass of insulin-producing pancreatic  $\beta$ -cells (Figure S1B) were observed in obese ZSF1 rats compared to lean controls, suggestive of insulin resistance. However, 14 weeks of nitrite administration had no effect on plasma insulin levels or  $\beta$ -cell mass in the ZSF1 rats (Figures 3A and S1B).

We next hypothesized that nitrite may increase sensitivity to insulin in skeletal muscle, which is the major tissue for insulin-stimulated glucose utilization. As shown in Figure 3B, phosphorylation of Akt, the central mediator of insulin signaling in skeletal muscle, was not altered in nitrite-treated or untreated obese ZSF1 rats compared to lean controls, suggesting the observed glucose lowering effect of nitrite is independent of insulin-receptor signaling. In addition to Akt, AMPK activation has been shown to increase glucose uptake in skeletal muscle in an insulin-independent manner<sup>46</sup>. We assessed the levels of AMPK activation in the skeletal muscle of these rats, collected after 14 weeks of nitrite supplementation, and

found a significant increase in AMPK phosphorylation in nitrite-treated obese rats compared to untreated animals (Figure 3C). In skeletal muscle, AMPK enhances glucose uptake by promoting GLUT4 translocation to the cellular membrane<sup>46</sup>. Analysis of GLUT4 levels in membrane protein extracts prepared from lean and obese rat skeletal muscles indicated that obese ZSF1 rats had lower levels of membrane-associated GLUT4 compared to lean rats and GLUT4 levels increased significantly following chronic nitrite treatment (Figure 3D). Together, our data suggest that prolonged nitrite supplementation increases muscle glucose uptake via insulin-independent AMPK activation and GLUT4 membrane translocation.

### **SIRT3 is required for the glucose lowering effect of nitrite in human skeletal muscle cells and in mice fed with a high-fat diet (HFD)**

To further explore mechanism(s) by which nitrite activates AMPK in skeletal muscle, we used human skeletal muscle cells cultured from muscle biopsies obtained from lean and obese volunteers. Cells were challenged with a combination of 0.2 mM palmitic acid, 25 mM glucose and 120 nM insulin (labeled as PGI), and a further short-term insulin stimulation (120 nM, 40 min) to induce insulin resistance (Figure S2A and S2B). Consistent with the effect of nitrite on AMPK activation in rat skeletal muscles, prolonged supplementation with 10  $\mu$ M nitrite increased AMPK phosphorylation (Figure 4A), accompanied by increased glucose uptake in primary human skeletal muscle cells (Figure 4B). Given that LKB1 and CaMKII are known upstream activators of AMPK<sup>31, 32</sup>, we investigated whether LKB1 and CaMKII are involved in nitrite-mediated AMPK activation. As shown in Figure 4C and S2E–G, phosphorylation levels of LKB1 and CaMKII were not altered in nitrite-treated or untreated human skeletal muscle cells, while the downstream ACC phosphorylation mirrored AMPK activation, suggesting LKB1 and CaMKII are not required for the activation of AMPK by nitrite. More recently, SIRT3 was recognized as an important regulator for AMPK activation in skeletal muscle<sup>35, 36</sup>. Since SIRT3 deficiency has been linked to the development of metabolic syndrome and PAH<sup>47, 48</sup>, we next assessed the activation levels of SIRT3 in human skeletal muscle cells. Increased SIRT3 activation levels (the antibody we used specifically recognizes the short active form of SIRT3 at ~28kDa, which contains the catalytic domain and regulates deacetylation) was observed in nitrite-treated human skeletal muscle cells (Figure 4D), concomitant with decreased acetylation of several mitochondrial proteins (Figure 4E), suggesting nitrite regulates muscle glucose uptake through SIRT3-AMPK activation. In order to further provide evidence for this observation, we used siRNA-mediated knockdown (KD) of SIRT3 in human skeletal muscle cells to examine the effect of reduced SIRT3 on AMPK activation and glucose uptake. Data presented in Figure 5A showed a ~85% baseline reduction of SIRT3 in the SIRT3 KD cells. Nitrite-treated SIRT3 KD cells had ~45% lower levels of AMPK phosphorylation compared to nitrite-treated control cells (Figure 5A), with correlated lower glucose uptake levels (Figure 5B), indicating that SIRT3 is required for nitrite-mediated AMPK activation and muscle glucose uptake.

We further studied the impact of SIRT3 deficiency on the glucose lowering effect of nitrite by exposing WT and SIRT3 KO mice to a HFD, which has been well described to induce glucose intolerance and insulin resistance<sup>49</sup>, in the presence or absence of nitrite (50 mg/L, in drinking water) (Figure 5C). We observed no difference in body weight and 14 hours



fasting blood glucose levels in 129-WT and SIRT3 KO mice (Figure S3B and S3C). However, the effect of nitrite in lowering blood glucose during the oral glucose tolerance test was completely abolished in SIRT3 KO mice compared to WT mice fed with a HFD (Figure 5D). These data support our finding that SIRT3 is required for the glucose lowering effect of nitrite.

### **Nitrite-mediated SIRT3 activation requires reactive oxygen species (ROS) generation**

We next examined the mechanism by which nitrite activates SIRT3. Since peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC1 $\alpha$ ) is a known transcription factor that regulates SIRT3 expression<sup>50</sup>, we first measured the transcript levels of PGC1 $\alpha$  and SIRT3 in human skeletal muscle cells. As shown in Figure S4A and S4B, both PGC1 $\alpha$  and SIRT3 mRNA levels were not increased in nitrite-treated cells, while the activation level of SIRT3 was increased (Figures 4D). Thus, our data suggest PGC1 $\alpha$ -mediated transcriptional regulation is not required for the activation of SIRT3 by nitrite. We next hypothesized that nitrite may activate SIRT3 through reduction to NO. To test this, human skeletal muscle cells were incubated with the conventional NO scavenger, cPTIO, in the presence or absence of nitrite and/or PGI, for 1 and 4 days. As shown in Figure S4C, the ability of nitrite to activate SIRT3 was not altered (fold change relative to PGI alone) by the perturbation of cPTIO, suggesting that activation of SIRT3 by nitrite is independent of NO. This is consistent with the fact that these cells are under normoxic conditions and many of the enzymes known to reduce nitrite to NO are more effective under hypoxia. Given that an increase in ROS has been demonstrated to activate SIRT3, which leads to SOD2 deacetylation and catabolism of superoxide<sup>50</sup>, we next investigated the role of ROS in activation of SIRT3 by nitrite. Human skeletal muscle cells were incubated with ROS scavengers, peg-catalase and peg-SOD (CAT/SOD), in the presence or absence of nitrite and/or PGI, for 1 and 4 days. Our data showed that the ability of nitrite to activate SIRT3 was reduced (fold change relative to PGI alone) with the duration of CAT/SOD treatment (Figure 4F). Taken together, these data suggest that nitrite reactions generate ROS that is necessary for SIRT3 activation.

### **Skeletal muscle SIRT3 activation is increased by chronic oral nitrite treatment in obese ZSF1 rats as well as in human volunteers with metabolic syndrome**

Consistent with our observations in human skeletal muscle cells, we found elevated activation levels of SIRT3 in the skeletal muscle of obese ZSF1 rats collected after 14 weeks of oral nitrite supplementation (Figure 6A). In addition, skeletal muscle samples obtained from human volunteers with metabolic syndrome without previous treatment history (BMI > 32 kg/m<sup>2</sup>) after 12 weeks of combined oral sodium nitrite (20 mg, twice daily) and sodium nitrate (1000 mg, once daily) treatment displayed even higher expression of active SIRT3 (Figure 6B). In line with increased SIRT3 activation, AMPK activation levels were also higher after combined nitrite/nitrate supplementation (Figure 6B).



## Early preventative treatment with oral nitrite and oral metformin normalize PH-HFpEF associated with metabolic syndrome via activation of SIRT3 and AMPK in SU5416/ZSF1 rats

To evaluate the effect of chronic oral supplementation of nitrite on PH-HFpEF associated with metabolic syndrome, we evaluated preventative treatment at the time of SU5416 exposure to the obese ZSF1 rat, as well as reversal treatment following late stage disease in our “two-hit” SU5416/ZSF1 rat model of PH-HFpEF. For the early phase preventative intervention study, two doses of nitrite (50 and 100 mg/L) were provided in drinking water for 14 weeks after a single injection of SU5416 to eight-week old obese ZSF1 rats (Figure 7A). Note that these rats already have metabolic syndrome at this time point but have not yet developed PH-HFpEF. The effect of nitrite on PH-HFpEF was compared with the canonical AMPK activator, metformin (300 mg/kg). Our data showed that both oral nitrite and oral metformin treatments lowered the increased right ventricular systolic pressure in SU5416/ZSF1 rats (Figure 7B), but had no effect on systemic blood pressure and LV and RV hypertrophy (Figure S5E–G). In addition, nitrite and metformin treatments resulted in lower percentage of media wall thickness as compared to untreated SU5416/ZSF1 rats (Figure 7C). For the late stage disease reversal study, nitrite (100 mg/L) and metformin (300 mg/kg) were given in drinking water 3–4 weeks after a single injection of SU5416 (100 mg/kg) to twenty-three-week old obese ZSF-1 rats, that have more advanced obesity, hyperglycemia, glucose intolerance, and insulin resistance (Figure S6A). Our data showed that both nitrite and metformin failed to reverse the increased right ventricular systolic pressure (Figure S6B) and were unable to reverse biventricular hypertrophy in the late phase model of PH-HFpEF (Figure S6C–D), suggesting that an early intervention of nitrite and metformin may be needed for the clinical treatment of PH-HFpEF.

We next examined the mechanism by which nitrite and metformin reduce pulmonary pressure and pulmonary vascular remodeling, as observed in the “early phase intervention” study. Although SIRT3 activation was not detected in pulmonary vessels obtained from SU5416/ZSF1 rats following nitrite and metformin treatments (Figure S7A), an increase in AMPK activation levels was observed, as AMPK phosphorylation signals co-localize with the smooth muscle layer (Figure 8A). In addition, nitrite and metformin increased SIRT3 activation levels in skeletal muscle (Figure 8B), but not in lung, LV, RV, and liver obtained from SU5416/ZSF1 rats after 14 weeks of oral supplementation (Figure S7B–E), accompanied by increased AMPK activation levels in skeletal muscle (Figure 8C). Note that SIRT3 KO mice fed with a HFD did not develop pulmonary hypertension (Figure S3D) so we were not able to confirm the role of SIRT3 on nitrite-mediated inhibition of pulmonary vascular remodeling in these experiments. Together, our data show that the early chronic oral supplementations of nitrite and metformin normalize PH-HFpEF associated with metabolic syndrome at least in part via SIRT3 and AMPK activation.

## Discussion

In this study, we developed a “two-hit” model of PH-HFpEF, which combines endothelial injury in rats with multiple features of metabolic syndrome. This model uncovers evidence of a relationship between pulmonary endothelial injury and severe metabolic syndrome,

which leads to the development of PH-HFpEF. Clinically, PH-HFpEF patients have elevated left ventricular end diastolic pressures, right ventricular systolic pressure, and right atrial pressure, preserved left ventricular ejection fraction, and biventricular hypertrophy, all features which were observed in our model. Using this model of PH-HFpEF associated with metabolic syndrome, we show that early chronic oral supplementations of nitrite and metformin reduce pulmonary pressures and vascular remodeling by a mechanism at least in part involving skeletal muscle SIRT3-AMPK activation and improved glucose uptake and metabolism. These findings are of significant importance, as there are no approved specific treatments for PH-HFpEF. In fact, the utilization of FDA-approved therapies for PAH in PH-HFpEF patients has been shown to worsen symptoms or cause adverse events <sup>3, 51</sup>.

The effects of SU5416-induced pulmonary endothelial injury and elevated pulmonary pressure in rats with metabolic syndrome observed in this study provides support for the proposed role of metabolic syndrome as a risk factor for the development of PH-HFpEF <sup>52-55</sup>. Epidemiological studies indicate that PH-HFpEF patients are older and have a higher prevalence of hypertension, diabetes, obesity, and coronary artery disease than patients with PAH <sup>3-6</sup>. Metabolic syndrome is associated with LV diastolic dysfunction and is considered a risk factor for PH-HFpEF. According to Robbins et al., more than 90% of patients with PH-HFpEF in their study had two or more features of metabolic syndrome <sup>6</sup>. Leung et al. also demonstrated that obesity is associated with worsening of LV diastolic dysfunction and elevated pulmonary pressures <sup>56</sup>.

Although oral supplementation of nitrate, the dietary precursor of nitrite, has been recently reported to reverse features of metabolic syndrome in eNOS knockout mice <sup>20</sup>, the mechanism of nitrate's effect and the role of its active metabolite nitrite on metabolic syndrome has not been established. Here, we show that chronic treatment with nitrite significantly reduces blood glucose levels and improves glucose intolerance in ZSF1 rats with severe metabolic syndrome. Unlike dietary nitrate treatment, which appeared to reduce body weight, improve glucose and lipid homeostasis, and decrease blood pressure in eNOS-deficient mice <sup>20</sup>, nitrite did not have any effect on weight loss and lipid homeostasis in obese ZSF1 rats. Mice with eNOS deficiency present a pre-diabetic phenotype with moderate insulin resistance, hyperlipidemia and hypertension <sup>11</sup>. In contrast, obese ZSF1 rats develop more profound diabetes associated with hyperglycemia, dyslipidemia, and hypertension at 8 weeks of age (the starting point of our study) <sup>57</sup>. Even though these metabolic defects become more severe as disease progresses, our data demonstrate that nitrite is still able to improve hyperglycemia and glucose intolerance over 14 weeks.

Glucose uptake is mainly controlled by two processes, insulin-dependent and insulin-independent signaling pathways. Normally, insulin binds to its receptor followed by Akt phosphorylation, which leads to GLUT4 membrane translocation for glucose uptake into skeletal muscle. Under insulin resistant and insensitive states, as observed in obese ZSF1 rats, Akt is not activated compared to controls and was not affected by nitrite treatment. These data suggest that the glucose lowering effect of nitrite is independent of insulin signaling. AMPK activation is critical for insulin-independent glucose uptake in skeletal muscle through GLUT4 membrane translocation <sup>46</sup>. Low AMPK activity due to nutrient overload and lack of exercise has been correlated with obesity and insulin resistance <sup>22-25</sup>.

Here, we show that AMPK activation is severely impaired in obese ZSF1 rats, accompanied by decreased GLUT4 membrane translocation. Chronic oral nitrite supplementation, in turn, increases AMPK phosphorylation and restores GLUT4 localization to the membrane, which is consistent with previous studies<sup>58, 59</sup>. Thus, AMPK activation appears to be an important mechanism behind the observed effect of nitrite on improving glucose uptake. Our finding may explain, at least in part, the underlying mechanism for nitrate-mediated improvement of glucose metabolism in the background of eNOS deficiency<sup>20</sup>. Additionally, AMPK is known to regulate glucose homeostasis by inhibiting hepatic glucose production<sup>60</sup>. Our data, however, showed that AMPK activation was not altered by nitrite treatment in the livers from rats with metabolic syndrome (Figure S8), suggesting that AMPK-GLUT4-mediated skeletal muscle glucose uptake is the primary mechanism by which nitrite improves hyperglycemia.

SIRT3 is a member of the sirtuin family of protein deacetylases that is preferentially localized in mitochondria and regulates reactive oxygen species (ROS) levels and global respiration, via target protein deacetylation<sup>50, 61, 62</sup>. It has been recently recognized as an important regulator of skeletal muscle metabolism and AMPK activation<sup>35, 36</sup>. Using SIRT3 knockdown in human skeletal muscle cells, we demonstrated that SIRT3 regulates AMPK activation and glucose uptake, an effect which is further confirmed in nitrite-treated SIRT3 deficient mice fed with a HFD. Hence, our data provide evidence that SIRT3 is linked to the glucose lowering effect of nitrite. The next question we answered is how SIRT3 is regulated by nitrite. Our data indicate that nitrite-mediated skeletal muscle SIRT3 activation is independent of NO, but at least in part dependent on ROS generation. This observation is consistent with previous findings that nitrite mediates delayed cytoprotection after ischemia/reperfusion through ROS-induced AMPK activation in a nitrite-dependent manner<sup>30</sup>. Our observation also confirms the previous findings that SIRT3 is activated by increase in ROS, that serve to detoxify ROS via target proteins (i.e. SOD2) deacetylation<sup>50</sup>. Exercise training has been shown to generate low levels of ROS. In addition, ROS has been reported to be involved in the activation of insulin-independent glucose uptake via AMPK-GLUT4 signaling during exercise, and this skeletal muscle contraction-stimulated glucose uptake can be elevated by up to 50-fold during maximal exercise in humans<sup>63, 64</sup>. In animals, exercise training has also been shown to increase SIRT3 expression<sup>35, 65</sup>. These studies suggest that nitrite activates ROS-signaling pathways similar to effects of exercise training. Further experiments are required to better understand the mechanism in this context.

Several recent studies have suggested that skeletal muscle insulin resistance and glucose intolerance, as well as global mitochondrial abnormalities, are the likely causes of severe exercise intolerance and worsening of functional capacity in patients with either metabolic syndrome, HFpEF, or PAH<sup>66–69</sup>. Exercise training leads to multiple health benefits within the cardiovascular and the musculoskeletal systems, and has been shown to improve metabolic syndrome, HFpEF, or PAH<sup>35, 70, 71</sup>. As stated above, exercise training increases SIRT3, which has been shown to modulate diabetes through maintaining of skeletal muscle insulin action and glucose disposal, and mitochondrial function<sup>36, 49, 72</sup>. Therefore, our observations suggest that activation of SIRT3, even at the level of skeletal muscle, may be crucial in regulating the beneficial actions of nitrite and metformin on treating PH-HFpEF,

as observed in our prevention studies. Although SIRT3 KO mice have been shown to develop spontaneous pulmonary hypertension<sup>48</sup> and HFD has been reported to induce pulmonary hypertension in apoE KO mice<sup>55</sup>, we found that 20 weeks of HFD treatment did not induce pulmonary hypertension in SIRT3 KO mice (Figure S3D). This may be due to higher resistance of the background strain 129/Sv mice to HFD or a longer period of HFD treatment is required to develop PH-HFpEF. It is also possible that a second hit (i.e. SU5416) is required to promote PH-HFpEF in our SIRT3 KO mice. Future studies are needed to determine whether SIRT3 is indeed required for the beneficial effect of nitrite, as well as metformin, on limiting PH-HFpEF.

Although marked glucose lowering effect of nitrite was observed only at week 7, but not for the entire 14-week exposure period, an increase in plasma adiponectin levels was observed in SU5416/ZSF1 rats following the 14 weeks of nitrite treatment (Figure S5). In comparison, metformin effectively improved hyperglycemia and glucose intolerance, and elevated circulation levels of adiponectin in SU5416/ZSF1 rats (Figure S5). As reported previously, adiponectin is an anti-diabetic adipokine secreted by adipose tissue and has been recognized as a key protector of both metabolic and vascular diseases<sup>73, 74</sup>. High levels of adiponectin have also been shown to suppress proliferation of pulmonary arterial smooth muscle cells, thus reversing PAH through AMPK activation<sup>55, 75</sup>. Our results showed that oral nitrite and metformin treatments resulted in higher AMPK activation in pulmonary vessels of SU5416/ZSF1 rats together with higher skeletal muscle SIRT3-AMPK activation and circulating adiponectin levels, suggesting that nitrite and metformin may alleviate PH-HFpEF through a broader set of mechanisms contributed by multiple organs, such as skeletal muscle, adipose tissue, and pulmonary vasculature. Aside from the proposed cross-organ communication, it is possible that other unknown substrates of nitrite and metformin or other proteins of the sirtuin family may directly activate AMPK in pulmonary vessels, thereby preventing the development of PH-HFpEF in SU5416/ZSF1 rats. The relative contribution of skeletal muscle and/or adipose tissue to the treatment of PH-HFpEF, compared to that of lung signaling events, needs to be further investigated.

We are intrigued by the lack of beneficial effects of nitrite and metformin in the more severely affected and older SU5416/ZSF1 rats (Figure S6). These findings may suggest that PH-HFpEF cannot be treated or reversed once it is firmly established and is resistant to the beneficial effect of nitrite and metformin on glucose homeostasis and insulin sensitivity. These findings suggest that earlier intervention with nitrite and metformin may be required for the treatment of PH-HFpEF, for example in patients presenting with signs of diabetes, insulin resistance, or impaired glucose metabolism with borderline PH severity determined by echocardiogram or right heart catheterization. In addition, our observations open a new avenue for combination therapy of nitrite and metformin in the management of PH-HFpEF.

In conclusion, we show that nitrite has similar effects to metformin, activating SIRT3-AMPK in skeletal muscle and enhancing insulin-independent muscle glucose uptake by stimulation of GLUT4 membrane translocation. We also demonstrate that chronic oral nitrite treatment activates skeletal muscle SIRT3-AMPK signaling in rats and humans, and to some extent, improves metabolic syndrome and cardiopulmonary hemodynamics in the

rat model of PH-HFpEF. Hence, future trials of chronic oral nitrite and oral metformin therapies may be considered for patients with PH-HFpEF.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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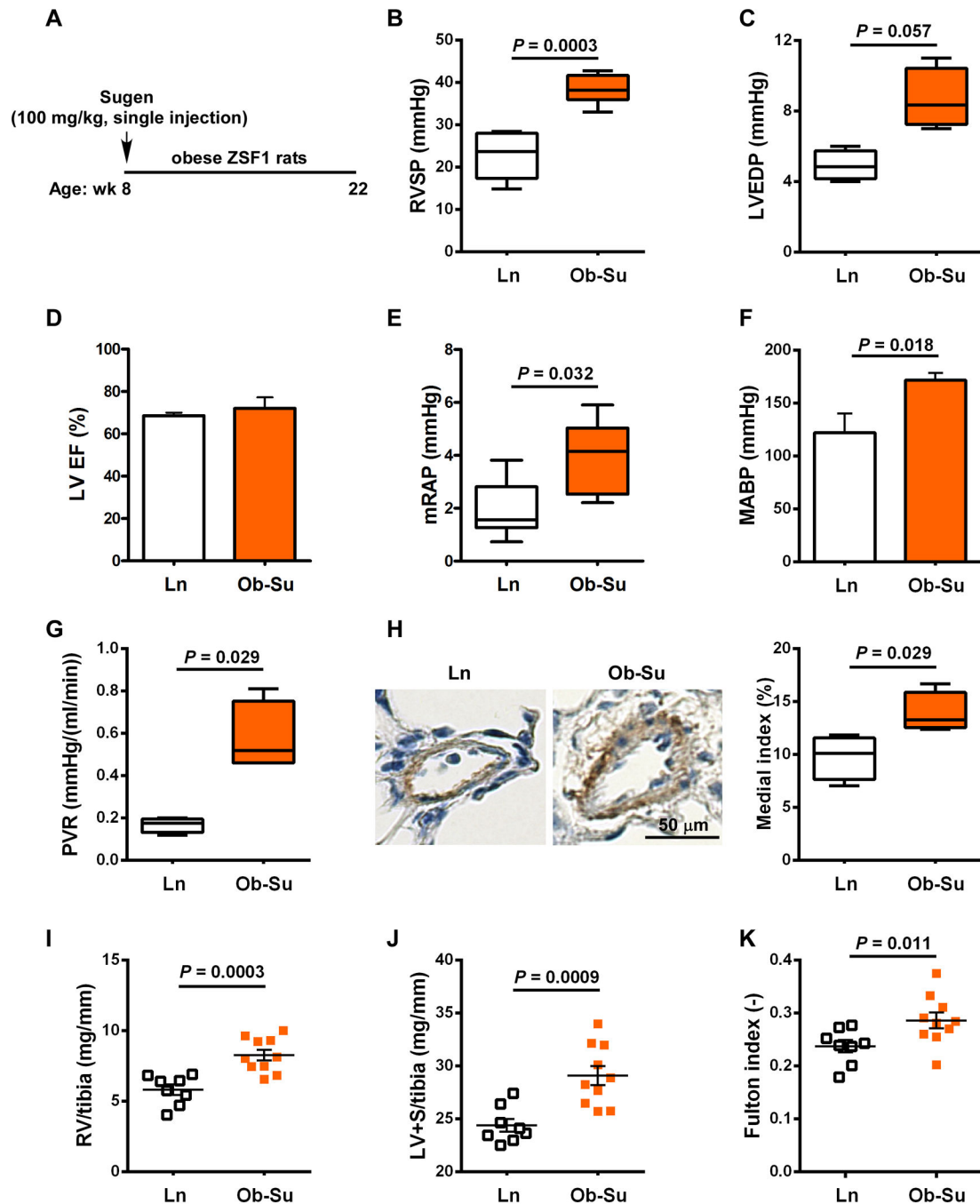


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### Clinical Perspectives

Pulmonary hypertension associated with heart failure with preserved ejection fraction (PH-HFpEF) represents the most frequent cause of PH worldwide, with no currently approved therapies. A major risk factor for the development of PH-HFpEF is metabolic syndrome. Although at present there are as many as nine FDA-approved drugs to treat pulmonary arterial hypertension (PAH) available on the market, all clinical trials with PAH drugs have targeted patients with normal left ventricular filling pressures and are contraindicated in patients with PH-HFpEF. In this study, we show that oral treatment with nitrite or metformin targets both the metabolic syndrome and the pulmonary vasculature, improving insulin sensitivity and reducing pulmonary pressures and vascular remodeling in a novel rat model of PH-HFpEF. These effects are caused by direct action on skeletal muscle, with activation of SIRT3-AMPK-GLUT4, which enhances skeletal muscle glucose uptake. We also confirm in patients with metabolic syndrome that chronic oral therapy with nitrite and nitrate similarly increases skeletal muscle SIRT3 and AMPK activation. Thus, our study suggests a potential role for nitrite and metformin as preventative treatments for PH-HFpEF developing in patients with metabolic syndrome and identifies skeletal muscle SIRT3 as a potential therapeutic target in the management of PH-HFpEF. Future trials of chronic oral nitrite and oral metformin, alone or in combination, may be considered for patients with PH-HFpEF.



**Figure 1.**

Development of a novel rat model of PH-HFpEF. **A**, A single subcutaneous injection of SU5416 (Sugen, 100 mg/kg) was administered to eight-week old obese ZSF1 rats. Fourteen weeks after SU5416 administration, **B–G**, right ventricular systolic pressure (RVSP, **B**), left ventricular end diastolic pressure (LVEDP, **C**), left ventricular ejection fraction (LVEF, **D**), mean right atrial pressure (mRAP, **E**), mean arterial blood pressure (MABP, **F**), and pulmonary vascular resistance (PVR, **G**) were measured. **H**, Medial index (%) were calculated ( $n = 5$ ). **I–K**, RV (**I**) and LV (**J**) mass normalized to tibial length and Fulton index

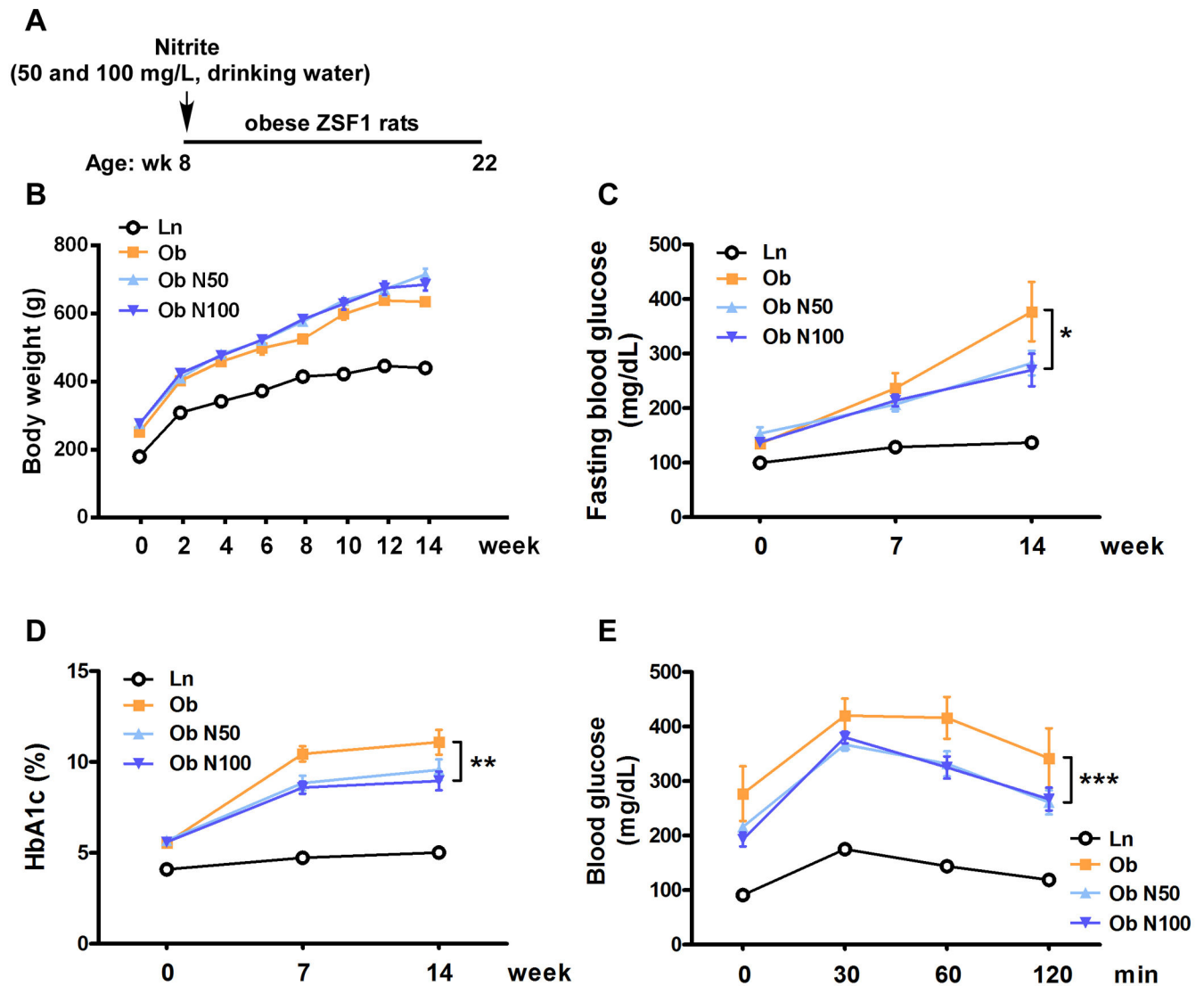
(K) were measured. Data are mean  $\pm$  SEM. Mann-Whitney U test was used for two-group comparison.

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**Figure 2.**

Chronic oral nitrite treatment improves hyperglycemia and glucose intolerance in obese ZSF1 rats. **A**, Two doses of nitrite (50 and 100 mg/L) were given in drinking water chronically for 14 weeks to obese ZSF1 rats (Ob). **B**, Body weights were measured during a 14-week period,  $n = 3-8$  rats per group. **C**, Fasting blood glucose levels were measured in whole blood samples collected at week 0, 7 and 14 weeks. Both Ob N50 and Ob N100 groups are associated with significant lower blood glucose levels compared to Ob alone (mixed effect model with bootstrapping:  $^*P = 0.048$  and  $0.016$ , respectively). **D**, HbA1c levels were measured in whole blood samples collected at week 0, 7 and 14 weeks. Both Ob N50 and Ob N100 groups are associated with significant lower HbA1c levels compared to Ob alone (mixed effect model with bootstrapping:  $^{**}P = 0.005$  and  $< 0.0001$ , respectively). **E**, At week 14, rats were challenged with oral glucose (2 g/kg) and tail-vein blood was sampled for glucose at the indicated times. Both Ob N50 and Ob N100 groups are associated

with significant lower blood glucose levels compared to Ob alone (mixed effect model with bootstrapping: both at \*\*\* $P < 0.0001$ ). All data are presented as mean  $\pm$  SEM.

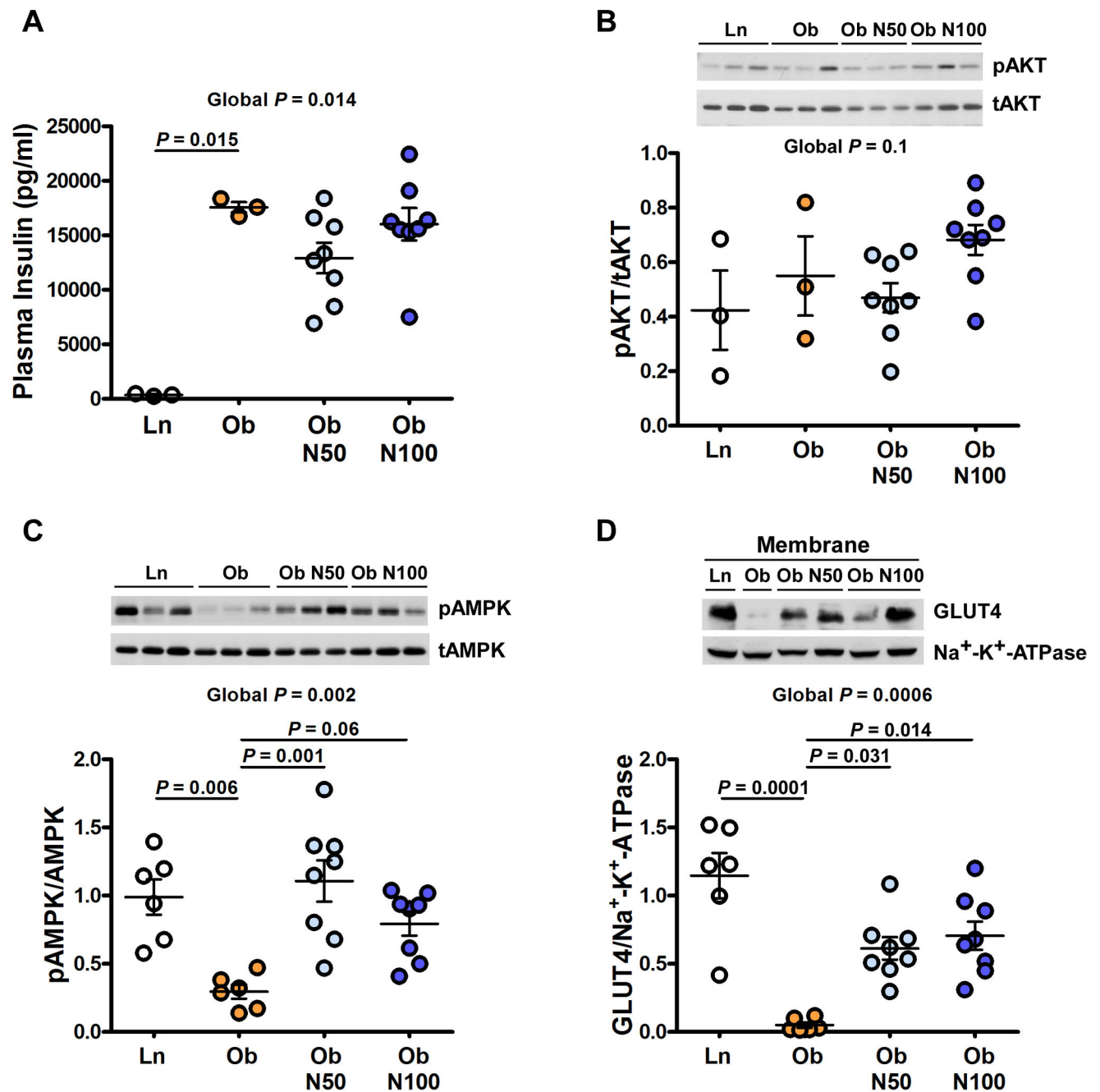
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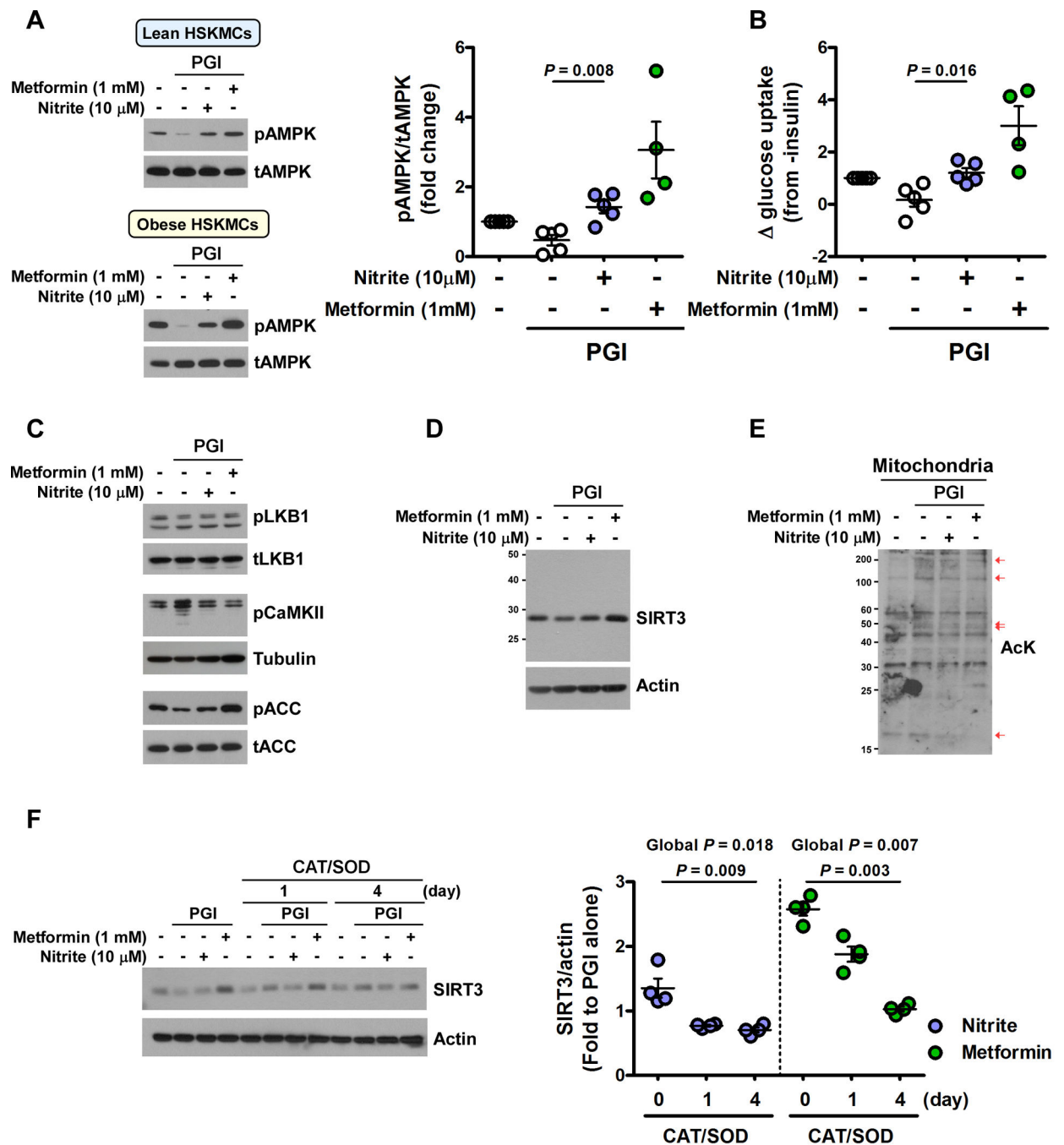




**Figure 3.**

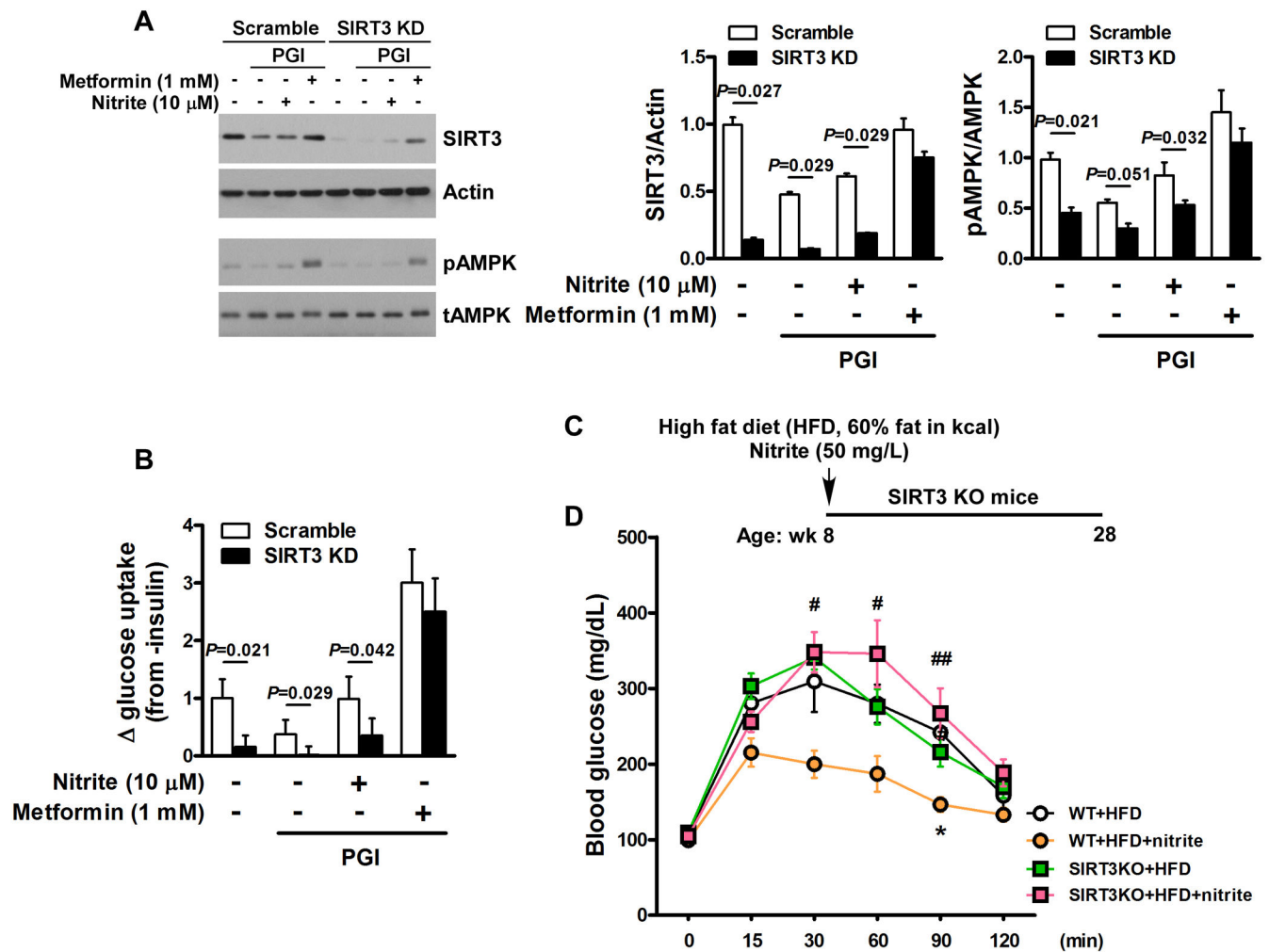
Chronic oral nitrite treatment improves glucose metabolism via AMPK phosphorylation and GLUT4 membrane translocation. At week 14, plasma samples and skeletal muscle were collected from lean and obese ZSF1 rats, treated or untreated with nitrite (50 and 100 mg/L). **A**, Plasma insulin levels were measured. **B–C**, Effects of nitrite on phosphorylation of insulin-dependent Akt signaling (**B**) and insulin-independent AMPK signaling (**C**) were detected by Western blot analyses in skeletal muscle. Each lane represents the skeletal muscle sample from an individual rat. The dot plots show pAkt/tAkt and pAMPK/tAMPK

ratio, accounting for Akt and AMPK activation, respectively. **D**, Representative Western blots for GLUT4 expression in membrane protein extracts from skeletal muscle. Equal membrane protein loading was ensured by examination of Na<sup>+</sup>-K<sup>+</sup>-ATPase. Global significance among four groups was determined by Kruskal-Wallis test, followed by *post-hoc* pairwise comparisons with the Dunn-Bonferroni procedure. All data are presented as mean ± SEM.

**Figure 4.**

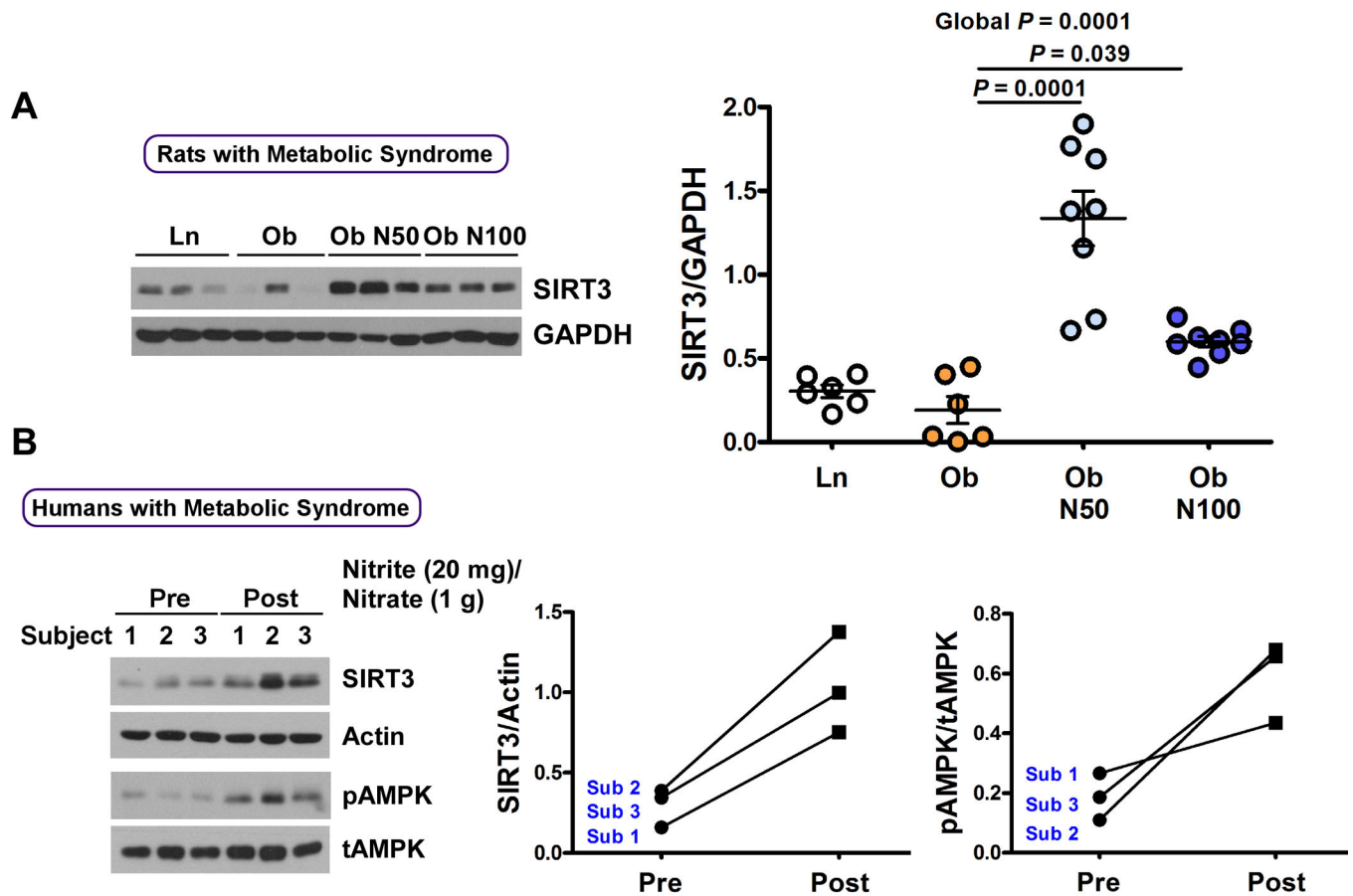
Nitrite increases activation of SIRT3 and AMPK in human skeletal muscles cells (HSKMCs). HSKMCs cultured from vastus lateralis muscles obtained from lean and obese volunteers were chronically treated with nitrite (10  $\mu$ M) and metformin (1 mM) throughout the differentiation period. These cells were then stimulated with 0.2 mM palmitic acid, 25 mM glucose and 120 nM insulin (PGI) for 24 hours, and a further short-term insulin stimulation (120 nM, 40 min) to induce insulin resistance. **A**, Representative Western blots for AMPK phosphorylation in HSKMCs obtained from lean and obese volunteers. Four to

five different biopsies were used for density analyses of Western blots for AMPK activation. Mann-Whitney U test was used to compare the effect of nitrite on AMPK activation in HSKMCs. **B**, Net glucose uptake was determined relative to basal uptake in HSKMCs. Mann-Whitney U test was used to compare the effect of nitrite on glucose uptake in HSKMCs. **C**, Phosphorylation and protein expression levels of upstream activators of AMPK (LKB1 and CaMKII) and downstream substrate of AMPK (ACC). **D**, Representative Western blot for activation levels of SIRT3. **E**, Western blot of protein acetylation in mitochondria isolated from HSKMCs. Red arrows show decreased acetylation of several mitochondrial proteins due to nitrite and metformin supplementations. **F**, HSKMCs were incubated with ROS scavengers, peg-catalase and peg-SOD (CAT/SOD, 50 U/ml each), in the presence or absence of nitrite and/or PGI for 1 and 4 days. Effect of ROS on SIRT3 activation was measured by Western blot. Dot plots show the fold change of activated SIRT3 relative to PGI alone. Decrease in SIRT3 activation levels correlates to the duration of CAT/SOD treatment in either nitrite or metformin group was determined by Kruskal-Wallis test, followed by *post-hoc* pairwise comparisons with the Dunn-Bonferroni procedure. All data are presented as mean  $\pm$  SEM.



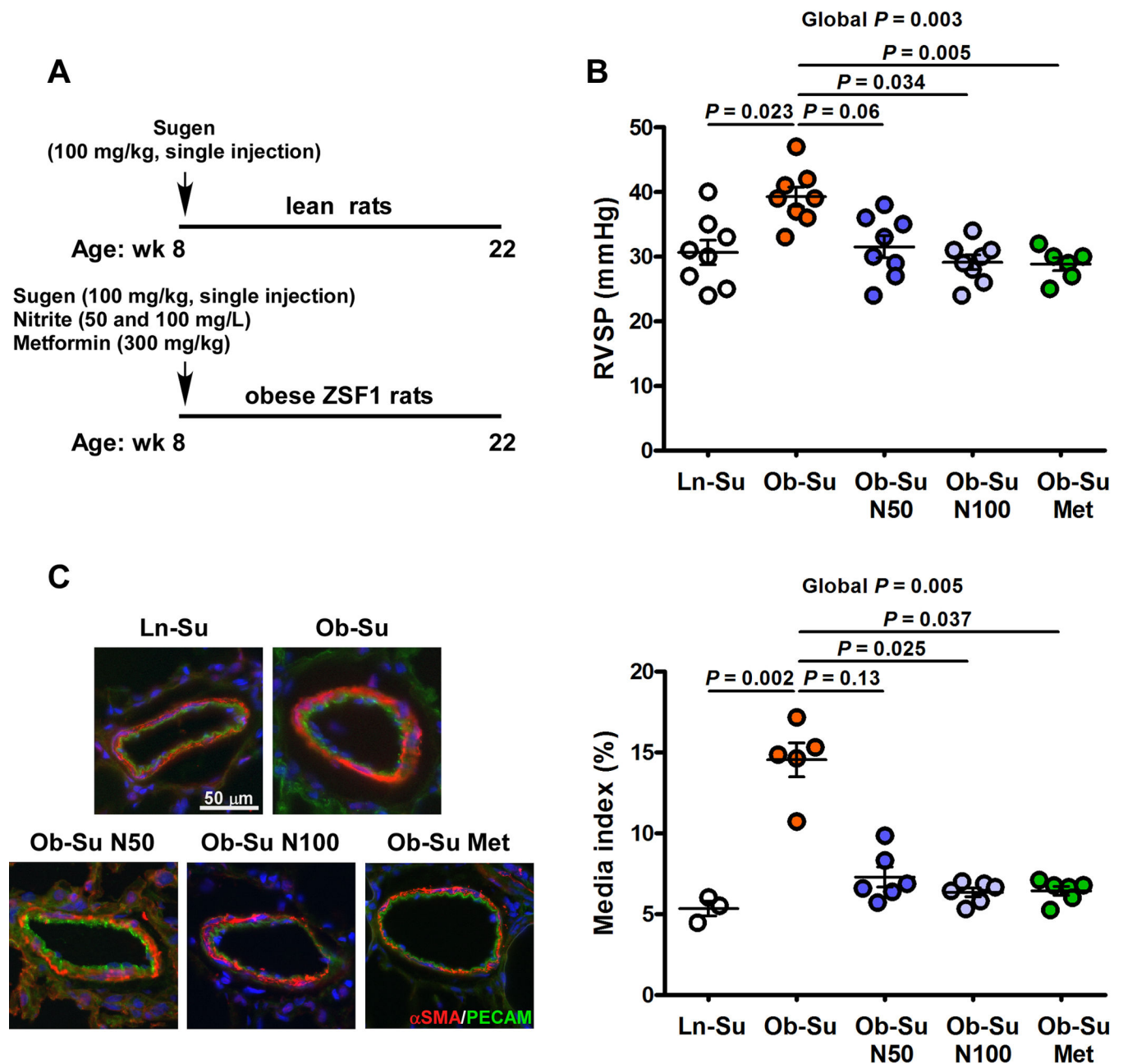
**Figure 5.**

SIRT3 is required for the glucose lowering effect of nitrite in HSKMCs and in mice fed a high-fat diet (HFD). **A**, HSKMCs were chronically treated with nitrite (10  $\mu$ M) and metformin (1 mM) throughout the differentiation period. After cells were differentiated to 70% confluence, cells were transiently transfected with siRNA targeting SIRT3 or scrambled control 48 hours before stimulation with PGI. Effect of SIRT3 knockdown (KD) on nitrite-mediated AMPK activation was measured by Western blots. Four different biopsies were used for density analyses of Western blots for SIRT3 and AMPK activation. **B**, Net glucose uptake was determined relative to basal uptake in HSKMCs ( $n = 4$ ). Mann-Whitney U test was used to compare two groups. **C**, WT and SIRT3 KO mice were fed with a HFD in the presence or absence of nitrite (50 mg/L, in drinking water) for 20 weeks. **D**, At week 20, mice were fasted for 6 hours and challenged with glucose (1.8 mg/g) and tail-vein blood was sampled for glucose at the indicated times. Kruskal-Wallis test was performed, followed by *post-hoc* pairwise comparisons with the Dunn-Bonferroni procedure at each time point. \* $P < 0.05$  nitrite-treated WT mice compared to WT mice fed with HFD alone; # $P < 0.05$  and ## $P < 0.01$  nitrite-treated SIRT3 KO mice compared to nitrite-treated WT mice fed with a HFD. All data are presented as mean  $\pm$  SEM.



**Figure 6.**

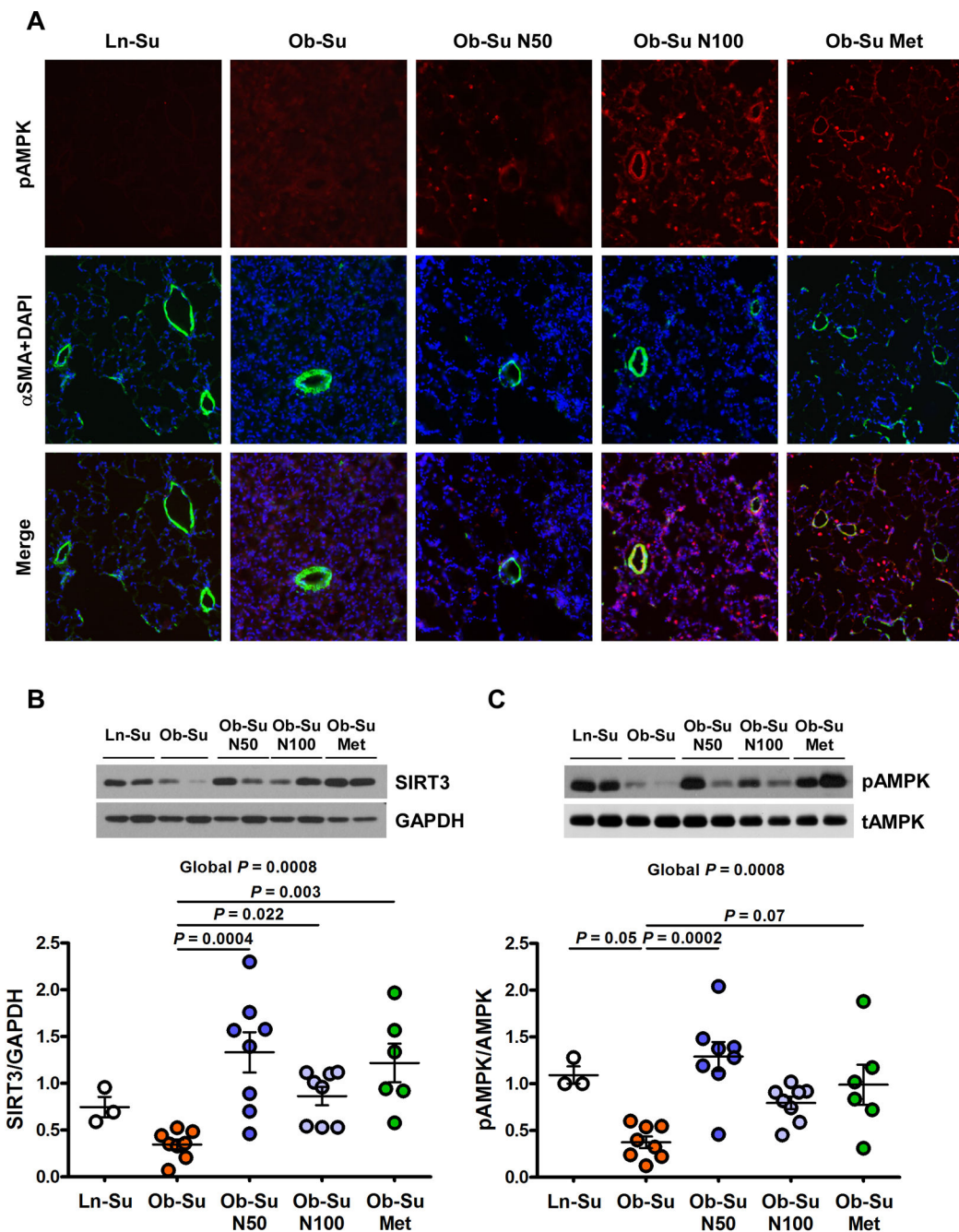
Increased SIRT3 activation levels in skeletal muscle obtained from obese ZSF1 rats and human patients with metabolic syndrome. **A**, Representative Western blot for SIRT3 activation levels in skeletal muscle obtained from lean and obese ZSF1 rats treated or untreated with nitrite (50 and 100 mg/L, in drinking water). Data are mean  $\pm$  SEM. Global significance among four groups was determined by Kruskal-Wallis test, followed by *post-hoc* pairwise comparisons with the Dunn-Bonferroni procedure. For comparison, AMPK activation levels are shown in Figure 3C. **B**, Representative Western blots and individual changes of SIRT3 and AMPK activation levels from pre-to-post (12 weeks) of combined nitrite/nitrate treatment in vastus lateralis muscle obtained from human patients with metabolic syndrome. Statistical differences were  $P = 0.028$  by paired *t*-test and  $P = 0.11$  by Wilcoxon signed-rank test for SIRT3 activation.  $P = 0.078$  by paired *t*-test and  $P = 0.11$  by Wilcoxon signed-rank test for AMPK activation.



**Figure 7.**

Early preventative treatment with oral nitrite and oral metformin treatments reduce pulmonary pressures and vascular remodeling in SU5416/ZSF1 rats. **A**, Nitrite (50 and 100 mg/L) and metformin (300 mg/kg) were given in drinking water chronically for 14 weeks to eight-week old SU5416/ZSF1 rats (Ob-Su). **B**, Right ventricular systolic pressures (RVSP) were measured. **C**, Representative images of lung sections stained with  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and quantification of medial index from the mean of 5 vessels per lung section from 3–6 rats per group. Data are mean  $\pm$  SEM. Global significance among five groups was determined by Kruskal-Wallis test, followed by *post-hoc* pairwise comparisons with the Dunn-Bonferroni procedure.





**Figure 8.**

Nitrite and metformin normalize PH-HFpEF associated with metabolic syndrome via activation of SIRT3 and AMPK. Nitrite (50 and 100 mg/L) and metformin (300 mg/kg) were given in drinking water chronically for 14 weeks to eight-week old SU5416/ZSF1 rats (Ob-Su). **A**, Representative images of lung sections stained with pAMPK,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and DAPI. **B–C**, Activation levels of SIRT3 (**B**) and AMPK (**C**) were analyzed by Western blot in skeletal muscles obtained from SU5416/ZSF1 rats. Data are

mean  $\pm$  SEM. Global significance among five groups was determined by Kruskal-Wallis test, followed by *post-hoc* pairwise comparisons with the Dunn-Bonferroni procedure.

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