Hypertrophic adipocyte-derived exosomal miR-802-5p contributes to insulin resistance in cardiac myocytes through targeting HSP60

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Abstract

Background: Epicardial adipose tissue (EAT) is implicated in insulin resistance, which has been recognized as a strongest predictor of the development of diabetic cardiomyopathy and subsequent heart failure. However, the underlying mechanism remains incompletely understood. Herein, we investigated the effect of hypertrophic adipocytes on cardiac insulin resistance.

Methods: Palmitate was used to induce hypertrophic 3T3-L1 adipocytes. Exosomes were purified from normal control or hypertrophic 3T3-L1 adipocyte-associated conditioned medium. Exosome-exposed neonatal rat ventricular myocytes (NRVMs) were treated with insulin to investigate the effects of exosomes on insulin signaling. Insulin sensitivity was evaluated by measuring insulin-stimulated Akt phosphorylation and glucose uptake. SiRNA techniques were used to downregulate protein levels and its efficiency was evaluated by western blot.

Results: Hypertrophic adipocyte-derived exosomes (h-Exo) induced insulin resistance in NRVMs. Furthermore, h-Exo high-expressed miR-802-5p. Insulin sensitivity of NRVMs was impaired by miR-802-5p mimic but improved by its inhibitor. TargetScan and luciferase reporter assays revealed that heat shock protein 60 (HSP60) was a direct target of miR-802-5p. Both h-Exo and miR-802-5p mimic could downregulate HSP60 protein levels. In addition, HSP60 silencing induced insulin resistance and mitigated the insulin-sensitizing effects of adiponectin. HSP60 depletion also significantly increased the expression levels of CHOP, a marker of the unfolded protein response (UPR), and enhanced oxidative stress, accompanied by the increased phosphorylation of JNK and IRS-1 Ser307. Inhibition of both miR-802-5p and endocytosis abolished the impacts of HSP60 knockdown on the UPR and oxidative stress.

Conclusion: Hypertrophic adipocyte-derived exosomal miR-802-5p caused cardiac insulin resistance in NRVMs through downregulating HSP60. These findings provide a novel mechanism by which EAT impairs cardiac function.

Background

Insulin resistance is a hallmark of obesity and the type 2 diabetes mellitus. Impaired insulin signaling in cardiac myocytes contributes to metabolic perturbations, which may adversely impact cardiac
structure and function, leading to the development of many different types of cardiovascular diseases such as diabetic cardiomyopathy and subsequent heart failure [1–3]. Underlying mechanisms include insulin resistance-induced mitochondrial dysfunction, endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), oxidative stress, and inflammation [1–3]. These abnormal pathophysiological processes finally lead to cardiac necrosis and apoptosis, fibrosis, hypertrophy, diastolic dysfunction and so on [1–3].

A growing number of evidences has indicated that the ectopic deposition of fat within and around the heart is closely associated with the impaired alterations in cardiac performance including cardiac oxidative stress, inflammation, and insulin resistance etc., all of those are responsible for cardiac energy metabolism disturbances that may finally lead to heart failure [4–6]. However, the molecular mechanisms underlying the impacts of ectopic fat accumulation on cardiac dysfunction are still required for further studies, although the abnormalities in cardiac structure and function are often attributed to lipotoxicity and adipokins produced and secreted by adipocytes [5–6].

Exosomes are small extracellular membrane vesicles with approximately 30–120 nm diameter size and released by almost all cell types [7]. Exosomes contain various biological substrates such as lipids, proteins, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and DNAs [7, 8]. These biological contents can be transferred to recipient cells and mediate intercellular communication under normal and pathological conditions [8]. Recently, adipocyte-derived exosomes are considered to be involved in development and progression of metabolic syndrome and its complications [9, 10].

HSP60 is a mitochondrial chaperone, which plays multiple roles in health and diseases through maintaining mitochondrial function and protein homeostasis [11]. Accumulating studies have evidenced the links between HSP60 dysfunction and cardiovascular diseases in the individuals with obesity and type 2 diabetes [12–14]. These subjects display high levels of serum (or circulating) HSP60 and down expression of intracellular HSP60 in cardiac cells [15–16], both of them have been proven to induce inflammation, mitochondrial dysfunction, oxidative stress, and even insulin resistance [12–14]. However, the mechanism by which the intracellular HSP60 is downregulated in cardiomyocytes remains unexplored.
Here, we show that hypertrophic 3T3-L1 adipocytes secreted exosomal miR-802-5p, which cause insulin resistance when administered to neonatal rat ventricular myocytes (NRVMs). Furthermore, we also found that miR-802-5p directly targeted HSP60 leading to the UPR and oxidative stress. These findings provide experimental explanation for obesity- or diabetes-induced cardiac insulin resistance.

**Materials And Methods**

**Antibodies and reagents**

Antibodies used in this study were obtained from Abcam (Cambridge, MA, USA) and Cell Signaling Technology (Billerica, MA, USA). Recombinant rat adiponectin globular form (Catalog#: SRP4593), insulin (91077C), and palmitic acid (P5585) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Cytochalasin D (Catalog#:1233) were acquired from R&D Systems, Inc (Minneapolis, MN, USA).

**Cell culture and treatment**

3T3-L1 preadipocytes (ATCC, CL-173) were cultured and differentiated according to standard protocols as our described previously [17, 18]. Adipocyte hypertrophy was induced by incubating fully differentiated 3T3-L1 adipocytes with 0.5 mmol/L palmitate (PA) for 48 h [19, 20]. PA was dissolved in ethanol and then associated with 20% FFA-free BSA [21]. The same concentration of ethanol mixed with 20% of FFA-free BSA was used as a control. To silence miRNA in hypertrophic adipocytes, 3T3-L1 adipocytes were incubated with lentiviruses containing miRNA inhibitor (antagomiRNA) for 6 h and then treated with 0.5 mmol/L PA for 48 h.

Isolation and culturing of neonatal rat ventricular myocytes (NRVMs) were performed as described previously [22, 23]. Briefly, hearts were rapidly and aseptically explanted from 1-to-2-day-old Sprague-Dawley rat pups. The ventricles were minced and then dissociated with 0.15% Collagenase Type I in calcium-free, magnesium-free HBSS for 1 h at 37 °C. The dissociated cells were pre-plated in regular culture dishes for 90 minutes to reduce non-myocytes. Cardiac myocytes were plated onto gelatin-coated culture dishes and incubated in DMEM medium containing 10% horse serum and 100 µmol/L bromodeoxyuridine (BrdU) for 48 h. Then, cells were grown in DMEM containing 5.5 mmol/L glucose, 20% fetal bovine serum (FBS), 100 µmol/L BrdU, and 1% penicillin/streptomycin at 5% CO₂ and 37 °C for 48 h before experiments.

To stimulate insulin signaling, the cells were starved serum for 18 h and then treated with 100 nmol/L
of insulin for 10 min [21, 24].

**Exosome isolation**
The cells were cultured in DMEM containing 10% exosomes-depleted FBS for 24 h. Exosome isolation and purification were carried out by differential ultracentrifugation [18, 25]. Purified exosomes were resuspended in PBS, further confirmed by identification of the exosome marker CD63 and fatty acid binding protein 4 (FABP4), and then stored at −80 °C pending for experiments. Total exosomes yield was determined by protein estimation using a BCA assay (Thermo Scientific, Rockford, IL, USA).

**MiR-802-5p quantification**
The organic extraction and purification of total RNA were carried out by using a Total Exosome RNA and Protein Isolation Kit from Life Technologies Corporation (#4478545, Grand Island, NY, USA). Quantitative real-time RT-PCR was performed as described previously [24, 26]. The miRNA primer kits were purchased from Ribobio (Guangzhou, China). Primer identification catalog numbers were 30442 for rno-miR-802-5p. Exogenous ath-miR-156a was considered an external reference. The 2-ΔΔCt method was used for relative quantification of gene expression.

**Determination of glucose uptakes**
Glucose uptake was evaluated using a Glucose Uptake Assay Kit (Colorimetric) (ab136955, Abcam, China) according to manufacturer's instructions.

**Plasmid construction and transfection**
Lentiviruses containing miR-802-5p mimic and inhibitor were obtained from GeneChem Biotechnology (Shanghai, China). To overexpress or silence miR-802-5p, the cells were incubated with serum-free medium containing lentiviruses for 6 h, and then grown in complete medium for another 36 h.

**Determination of intracellular ROS**
The real-time generation of ROS in the cells was determined by using OxiSelect™ Intracellular ROS Assay Kit (STA-342, Cell Biolabs, Inc. San Diego, CA, USA) according to the manufacture’s protocol. The fluorescence intensity was captured with fluorescence microscopy and quantified by automated image analysis (Image Pro Plus, Media Cybernetics, Rockville, MD, USA).

**SiRNA and transfection**
The small interfering RNA (siRNA) targeting rat HSP60 (Hspd1, NM_022229) was synthesized by QIAGEN China (Shanghai) Co. (Shanghai, China). Transfection was performed with 120 pmol/L of
siRNA using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Knockdown efficiency was assessed by western blot. The most effective sequences of siRNA and its paired control used in the experiments were as follows 5'-GAGAGGTGTGATGGCTTGAT-3' and 5'-GAGTGTGGTAGGGTTTGCTGAGAT-3'.

Determination of protein carbonyl groups and western blot
Protein carbonyl groups as a biomarker of oxidative stress were measured by a Protein Carbonyl Assay Kit (Western Blot) (ab178020, Abcam). Western blot was performed as described previously [17, 18, 21].

Statistical analysis
All data are expressed as the mean ± S.D. Differences between the groups were examined for statistical significance using analysis of variance (ANOVA), followed by a Newman-Keuls post hoc test. P < 0.05 was considered significant.

Results
Hypertrophic adipocyte-derived exosomes induced insulin resistance in NRVMs
To observe the impacts of exosome on insulin action, NRVMs were starved serum for 6 h and then treated with or without 50 µg/L of exosomes for 18 h, followed by stimulation with or without 100 nmol/L of insulin for 10 min. Meanwhile, the cells were pretreated with 2 µg/mL of cytochalasin D (CytoD), an endocytosis inhibitor for 30 min to confirm that these changes were caused by exosome uptake. Exosomes were purified from normal control or hypertrophic 3T3-L1 adipocyte-associated conditioned medium, respectively. As shown in Fig. 1, administration of hypertrophic adipocyte-derived exosomes (h-Exo) markedly suppressed insulin-stimulated phosphorylation of Akt T308, a site sensitizing to insulin stimulation, when compared with treatment with normal control adipocyte-derived exosome (nc-Exo) (Fig. 1a and b). Consistent with this result, h-Exo treatment also reduced insulin-stimulated glucose uptake (Fig. 1c). Importantly, these deleterious effects of h-Exo on insulin signaling were significantly mitigated by CytoD supplementation (Fig. 1), suggesting that h-Exo contributed to insulin resistance in NRVMs.

MiR-802-5p mediated exosome action on insulin signaling in NRVMs
Previous study has demonstrated that miR-802 levels in serum, liver, brown adipose tissues are significantly increased in C57BL/6J mice fed with high fat-high sucrose diet (HFHS) [27]. Therefore,
serum miR-802 has been recognized as a biomarker for type 2 diabetes [27]. It is interesting to note that mice fed with HFHS display an increase of miR-802 levels in white adipose tissues, although this change has no statistical significant due to a low n number for a sample (n = 3) [27]. In addition, palmitate has been found to enhance miR-802 expression in pancreatic β cells [28]. Hence, we determined whether the inhibitory effects of h-Exo on insulin signaling were caused by exosomal miR-802 from hypertrophic adipocytes. As expected, the miRNA sequencing analysis revealed that h-Exo contained high levels of miR-802-5p, when compared with nc-Exo (Fig. 2a).

Next, the specific exosomes (miR-inh-Exo) were prepared from palmitate-induced hypertrophic 3T3-L1 cells in which miR-802-5p was inhibited. When miR-inh-Exo-exposed NRVMs were starved serum for 18 h and then treated with 100 nmol/L insulin for 10 min, we found that inhibition of miR-802-5p significantly mitigated the inhibitory effects of exosome on Akt T308 phosphorylation and glucose uptake in NRVMs (Figure S1).

In addition, the impacts of miR-802-5p on insulin sensitivity were investigated in NRVMs. NRVMs transfected with miR-802-5p mimic, inhibitor (inh), and their paired controls were starved serum for 18 h and then stimulated with or without 100 nmol/L insulin for 10 min. we found that insulin-stimulated Akt T308 phosphorylation and glucose uptake were significantly decreased by miR-802-5p mimic (Fig. 2b-d) and increased by miR-802-5p inhibitor (Fig. 2e-g).

Taken together, these results indicated that exosomal miR-802-5p was responsible for the impairment of insulin signaling.

**MiR-802-5p downregulated HSP60 in NRVMs**

Bioinformatic prediction using TargetScan online software identified that Hspd1 is the direct downstream target of miR-802-5p (Fig. 3a). Therefore, luciferase reporter assay was performed in NRVMs transfected with plasmids containing predicted miR-802-5p-binding sites in 3’ untranslated regions (UTR). As shown in Fig. 3b, luciferase activity was rescued by mutation of the 3’UTR of rat Hspd1. Furthermore, h-Exo-reduced HSP60 protein levels in NRVMs were restored by inhibitions of both exosome uptake (Fig. 3c and d) and miR-802-5p (Fig. 3e and f). In addition, miR-802-5p mimic significantly downregulated HSP60 protein levels in NRVMs (Fig. 3g and h). These results demonstrate
that HSP60 was indeed a direct target of miR-802-5p.

**HSP60 deficiency induced insulin resistance in NRVMs**

To understand the functional role of HSP60 in regulating insulin sensitivity, NRVMs transfected with HSP60 siRNA or its paired control sequence were starved serum for 18 h and then stimulated with or without 100 nmol/L insulin for 10 min. As shown in Fig. 4, HSP60 silencing significantly inhibited insulin-stimulated Akt T308 phosphorylation (Fig. 4a and b) and glucose uptake (Fig. 4c), suggesting that loss of HSP60 impaired insulin action.

When HSP60 knockdown and control NRVMs were starved serum for 18 h, and then treated with or without 2 µg/mL globular adiponectin for 60 min, followed by stimulation with or without 100 nmol/L insulin for 10 min, we found that HSP60 depletion abolished the positively regulation of adiponectin on insulin-stimulated Akt T308 phosphorylation (Figure S2a and S2b) and glucose uptake (Figure S2c). Since adiponectin has been evidenced to sensitize insulin signaling [29], these result indicate that HSP60 depletion inhibited adiponectin action on insulin signaling.

**Loss of HSP60 induced UPR and ROS in NRVMs**

It has been documented that HSP60 plays a key role in maintaining mitochondrial function and protein homeostasis under stress conditions [11]. Therefore, we evaluated the effects of HSP60 knockdown on the UPR and ROS, both of them have been suggested to contribute to insulin resistance by activating JNK/IRS-1 signaling pathway [30, 31]. HSP60 silencing and control NRVMs were starved serum for 18 h. As shown in Fig. 5, HSP60 depletion significantly increased PERK phosphorylation and the protein levels of the UPR marker CHOP (Fig. 5a-c), enhanced intracellular ROS formation (Fig. 5d), and increased protein carbonylation (Fig. 5e), the latter is one of the most harmful irreversible oxidative protein modifications and considered as a major hallmark of oxidative stress-related disorders [32, 33]. Meanwhile, HSP60 knockdown significantly increased phosphorylation of JNK and IRS-1 Ser307 (Fig. 5f and g), suggesting that the UPR- and ROS-activated JNK/IRS-1 signaling mediated the inhibitory effects of HSP60 knockdown on insulin signaling.

Additionally, h-Exo-exposed NRVMs resulted in obvious increases in PERK phosphorylation and CHOP protein expression (Fig. 6a-c), ROS formation (Fig. 6d), and protein carbonylation (Fig. 6e). Similar
observations were made in NRVMs treated with miR-802-5p mimic (Fig. 6f-j). These h-Exo-induced changes were suppressed by endocytosis inhibitor CytoD (Fig. 6a-e). In addition, miR-inh-Exo-exposed NRVMs displayed obvious reductions in PERK phosphorylation and CHOP protein expression (Figure S2a-c), ROS formation (Figure S2d), and protein carbonylation (Figure S2e), when compared with h-Exo-exposed NRVMs. These results further suggest that exosomal miR-802-5p/HSP60 signaling was responsible for the enhancement of the UPR and ROS.

Discussion

Obesity- or diabetes-related epicardial adipose tissue (EAT), a form of visceral fat, has been implicated in the development and progression of various heart diseases including hypertensive heart disease, ischemic cardiomyopathy, diabetic cardiomyopathy and so on [34, 35]. EAT can supply free fatty acids for myocardial energy production. However, substantially increased EAT results in its pathophysiology changes leading to secretion of deleterious factors to cardiac myocytes, including “bad” adipokines, pro-inflammatory factors, and oxidative factors [34, 35]. All of them create a suitable environment for the development of heart diseases [4-6]. Currently, adipocyte-derived exosomes have been suggested to mediate the impaired effects of EAT on cardiac structure and function through releasing specific miRNAs. In mice fed with high-fat diet, miR-130b-3p from dysfunctional adipocyte-derived small extracellular vesicles exacerbates myocardial ischemia/reperfusion injury [36]. It is interesting to note that adipocyte-derived exosome functions as a mediator between adipocytes and insulin resistance [9, 37]. For example, adipocyte-derived exosomal miR-27a mediates obesity-triggered insulin resistance in skeletal muscle [37]. In the present study, we demonstrated that hypertrophic adipocyte-derived exosome induced insulin resistance in NRVMs (Fig. 1). Furthermore, miR-802-5p enriched in hypertrophic adipocyte-derived exosome and negatively regulated insulin sensitivity (Fig. 1 and Figure S1). Thus, consistent with previous studies, our findings indicate that hypertrophic adipocyte-derived exosomal miR-802-5p caused cardiac insulin resistance.

MiR-802 possesses multiple function. It has been reported to regulate cancer development, alleviate lipopolysaccharide (LPS)-induced acute lung injury, and modulate the expression of human
angiotensin II type I receptor [38–40]. In the term of metabolism, miR-802 impairs glucose metabolism and causes nephropathy in both obese mice and human [41, 42]. Thus, miR-802 is considered as a promising biomarker for obesity- or diabetic-related disorders [27]. In the present study, our findings identify that hypertrophic adipocyte-derived exosomal miR-802-5p functions as a key modulator for EAT-induced cardiac insulin resistance.

HSP60 has diverse effects on heart, which dependent on its location. HSP60 can be released into the extracellular space including serum by various cell types [43]. Extracellular HSP60, even at low concentration, causes cardiac myocyte apoptosis and necrosis [44]. The higher mean plasma levels of HSP60 are closely associated with clinically manifest cardiovascular diseases in the patients with type 1 or type 2 diabetes [45]. Additionally, the increased levels of anti-HSP60 antibody in the plasma is recognized as a risk factor for coronary heart disease and ischemic stroke [46, 47]. Hence, extracellular HSP60 is possibly dangerous to the cell function. Interestingly, the impacts of intracellular HSP60 on heart remains controversial. Transgenic HSP60 expression in the embryonic stage causes neonatal death in mice, accompanied with increased apoptosis and myocyte degeneration that possibly contributes to neonatal heart failure [48]. In contrast, intracellular HSP60 is low expressed in diabetic heart [49]. Decreased HSP60 inhibits insulin-like growth factor (IGF)-1 signaling pathway leading to the development of diabetic cardiomyopathy [49]. Furthermore, abnormal distribution of HSP60 on the cell surface trigger cell apoptosis leading to heart failure [50]. Loss of HSP60 in adult mouse hearts results in dilated cardiomyopathy, heart failure, and lethality [51]. But overexpression of HSP60 in NRVMs protects cardiac cells from apoptotic cell death induced by stress stimuli like ischemia and ischemia/reoxygenation [52, 53]. In present study, our results demonstrated that HSP60 silence induced insulin resistance in NRVMs (Fig. 4). Given that cardiac insulin resistance is an importantly promotive factor for diabetic cardiomyopathy [1–3], our findings indicate that HSP60 deficiency is a risk factor contributing to the development of diabetic cardiomyopathy.

HSP60 is a highly conserved mitochondrial chaperone responsible for the protein folding, transport, trafficking, and quality control of mitochondrial proteostasis. Under stressful conditions, the
abundance of HSP60 protein is compensatorily upregulated and increased HSP60 protects cells from oxidative stress, inflammation, and apoptosis. Therefore, loss of HSP60 will impaired mitochondrial function, which has been recognized as a primary abnormality contributing to the pathogenesis of cardiac insulin resistance and diabetic cardiomyopathy [2, 3]. In the present study, we found that HSP60 depletion significantly raised PERK phosphorylation and CHOP protein levels, increased intracellular ROS formation, and enhanced expression levels of protein carbonylation (Fig. 5a-d). These findings are consistent with previous study showing that knockdown of HSP60 in adult mouse hearts upregulates ROS production and increases CHOP mRNA levels at age of 9 weeks and 11 weeks [51], suggesting an impairment of mitochondrial function. It is well-known that excessive or prolonged UPR activation and ROS accumulation can trigger JNK/IRS-1 signaling pathway leading to insulin resistance [30, 31]. In the present study, the impacts of HSP60 knockdown on the UPR and ROS were accompanied with increased phosphorylation of JNK and IRS-1 S307, further confirming HSP60 knockdown-induced insulin resistance is dependent on its impairment on mitochondrial function.

Adiponectin, an adipokine produced by white adipose cells, has been proposed to treat obesity- or diabetes-related cardiomyopathy, at least partly through its insulin-sensitizing properties [29, 54–56]. In the present study, our results found that HSP60 depletion diminished the positive effects of adiponectin on insulin-stimulated Akt phosphorylation and glucose uptake (Figure S2), suggesting that HSP60 silencing resulted in adiponectin resistance. This inhibitory effects on adiponectin action may attribute to HSP60 knockdown-induced degradation of adiponectin receptor (Zhang D et al., Paper in press).

In the present study, like HSP60 knockdown, miR-802-5p mimic and hypertrophic adipocyte-derived exosome generated a similar promotion effect on the UPR and ROS (Fig. 6). Moreover, these changes were greatly abrogated by both inhibition of exosome uptake and deletion of miR-802-5p in adipocytes (Fig. 6 and S3). Therefore, hypertrophic adipocyte-derived exosome induced cardiac insulin resistance through exosomal miR-802-5p/HSP60 signaling pathway.

**Conclusion**

In summary, we demonstrated that hypertrophic adipocyte-derived exosomal miR-802-5p targeted
HSP60 leading to UPR activation and ROS accumulation, and ultimate cardiac insulin resistance (Fig. 7). These findings provide a novel mechanism by which EAT impairs cardiac function. These findings also indicate that miR-802-5p/HSP60 might represent a promising therapeutic opportunity in diabetic cardiomyopathy, although more in vitro and in vivo studies are necessary to further confirm our findings and to gain a full understanding of miR-802-5p/HSP60 relevance.

Abbreviations
Akt: Protein kinase B (PKB); AS160: Akt Substrate of 160 kDa; BSA: Bovine serum albumin; CHOP: C/EBP-homologous protein; CytoD: Cytochalasin D; 2-DG: 2-Deoxy-D-glucose; EAT: Epicardial adipose tissue; ER stress: endoplasmic reticulum stress; FABP4: fatty acid binding protein 4; FFA: Free fatty acid; HSP60: HBSS: Hanks’ Balanced Salt Solution; Heat shock protein 60; h-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes; inh: Inhibitor; INS: Insulin; IRS-1: insulin receptor substrate 1; JNK: c-Jun NH2-terminal kinase; miR: MicroRNA; miR-inh-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes with miR-802-5p knockdown; nc-Exo: Exosomes purified from normal control 3T3-L1 adipocytes; NRVMs: neonatal rat ventricular myocytes; PA: palmitate; PDK1: phosphoinositide dependent kinase-1; PERK: Protein kinase R-like endoplasmic reticulum kinase; ROS: Reactive oxygen species; siRNA: Small interfering RNA; UPR: Unfolded protein response.

Declarations

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Authors’s contributions

ZW and CW participated in the design of the study. ZW, JL, LL, YZ, and MK carried out the experiments, data analysis, and draft the manuscript. ZW and CW contributed in finalizing the manuscript. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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References
1. Riehle C, Abel ED. Insulin Signaling and Heart Failure. Circ Res. 2016;118(7):1151-69.
2. Jia G, DeMarco VG, Sowers JR. Insulin resistance and hyperinsulinaemia in diabetic cardiomyopathy. Nat Rev Endocrinol. 2016;12(3):144-53.
3. Jia G, Whaley-Connell A, Sowers JR. Diabetic cardiomyopathy: a hyperglycaemia- and insulin-resistance-induced heart disease. Diabetologia. 2018;61(1):21-8.
4. Piché ME, Poirier P. Obesity, ectopic fat and cardiac metabolism. Expert Rev Endocrinol Metab. 2018;13(4):213-21.
5. Wolf P, Winhofer Y, Krššák M, Krebs M. Heart, lipids and hormones. Endocr Connect. 2017;6(4):R59-R69.

6. Levelt E, Pavlides M, Banerjee R, Mahmod M, Kelly C, Sellwood J, Ariga R, Thomas S, Francis J, Rodgers C, Clarke W, Sabharwal N, Antoniades C, Schneider J, Robson M, Clarke K, Karamitsos T, Rider O, Neubauer S. Ectopic and Visceral Fat Deposition in Lean and Obese Patients With Type 2 Diabetes. J Am Coll Cardiol. 2016;68(1):53-63.

7. Samanta S, Rajasingh S, Drosos N, Zhou Z, Dawn B, Rajasingh J. Exosomes: new molecular targets of diseases. Acta Pharmacol Sin. 2018;39(4):501-13.

8. Gurunathan S, Kang MH, Jeyaraj M, Qasim M, Kim JH. Review of the Isolation, Characterization, Biological Function, and Multifarious Therapeutic Approaches of Exosomes. Cells. 2019;8(4). pii: E307.

9. Hubal MJ, Nadler EP, Ferrante SC, Barberio MD, Suh JH, Wang J, Dohm GL, Pories WJ, Mietus-Snyder M, Freishtat RJ. Circulating adipocyte-derived exosomal MicroRNAs associated with decreased insulin resistance after gastric bypass. Obesity (Silver Spring). 2017;25(1):102-10.

10. Ferrante SC, Nadler EP, Pillai DK, Hubal MJ, Wang Z, Wang JM, Gordish-Dressman H, Koeck E, Sevilla S, Wiles AA, Freishtat RJ. Adipocyte-derived exosomal miRNAs: a novel mechanism for obesity-related disease. Pediatr Res. 2015;77(3):447-54.

11. Deocaris CC, Kaul SC, Wadhwa R. On the brotherhood of the mitochondrial chaperones mortalin and heat shock protein 60. Cell Stress Chaperones. 2006;11(2):116-28.

12. Habich C, Sell H. Heat shock proteins in obesity: links to cardiovascular disease. Horm Mol Biol Clin Investig. 2015;21(2):117-24.

13. Juwono J, Martinus RD. Does Hsp60 Provide a Link between Mitochondrial Stress and Inflammation in Diabetes Mellitus? J Diabetes Res. 2016;2016:8017571.
14. Bellini S, Barutta F, Mastrocola R, Imperatore L, Bruno G, Gruden G. Heat Shock Proteins in Vascular Diabetic Complications: Review and Future Perspective. Int J Mol Sci. 2017;18(12). pii: E2709.

15. Grundtman C, Kreutmayer SB, Almanzar G, Wick MC, Wick G. Heat shock protein 60 and immune inflammatory responses in atherosclerosis. Arterioscler Thromb Vasc Biol. 2011;31:960-8.

16. Oksala NK, Laaksonen DE, Lappalainen J, Khanna S, Nakao C, Hanninen O, et al. Heat shock protein 60 response to exercise in diabetes: effects of alpha-lipoic acid supplementation. J Diabetes Complications. 2006;20:257-61.

17. Zhang D, Zhang Y, Ye M, Ding Y, Tang Z, Li M, Zhou Y, Wang C. Interference with Akt signaling pathway contributes curcumin-induced adipocyte insulin resistance. Mol Cell Endocrinol. 2016;429:1-9.

18. Wu Q, Li J, Li Z, Sun S, Zhu S, Wang L, Wu J, Yuan J, Zhang Y, Sun S, Wang C. Exosomes from the tumour-adipocyte interplay stimulate beige/brown differentiation and reprogram metabolism in stromal adipocytes to promote tumour progression. J Exp Clin Cancer Res. 2019;38(1):223.

19. Yeop Han C, Kargi AY, Omer M, Chan CK, Wabitsch M, O'Brien KD, Wight TN, Chait A. Differential effect of saturated and unsaturated free fatty acids on the generation of monocyte adhesion and chemotactic factors by adipocytes: dissociation of adipocyte hypertrophy from inflammation. Diabetes. 2010;59(2):386-96.

20. Takahashi K, Yamaguchi S, Shimoyama T, Seki H, Miyokawa K, Katsuta H, Tanaka T, Yoshimoto K, Ohno H, Nagamatsu S, Ishida H. JNK- and IκB-dependent pathways regulate MCP-1 but not adiponectin release from artificially hypertrophied 3T3-L1 adipocytes preloaded with palmitate in vitro. Am J Physiol Endocrinol Metab. 2008;294(5):E898-909.
21. Wang C, Liu M, Riojas RA, Xin X, Gao Z, Zeng R, Wu J, Dong LQ, Liu F. Protein kinase C theta (PKC\text{theta})-dependent phosphorylation of PDK1 at Ser504 and Ser532 contributes to palmitate-induced insulin resistance. J Biol Chem. 2009;284(4):2038-44.

22. Jiang P, Zhang D, Qiu H, Yi X, Zhang Y, Cao Y, Zhao B, Xia Z, Wang C. Tiron ameliorates high glucose-induced cardiac myocyte apoptosis by PKC\delta-dependent inhibition of osteopontin. Clin Exp Pharmacol Physiol. 2017;44(7):760-70.

23. Khan S, Zhang D, Zhang Y, Li M, Wang C. Wogonin attenuates diabetic cardiomyopathy through its anti-inflammatory and anti-oxidative properties. Mol Cell Endocrinol. 2016;428:101-8.

24. Li M, Zhang Y, Cao Y, Zhang D, Liu L, Guo Y, Wang C. Icariin Ameliorates Palmitate-Induced Insulin Resistance Through Reducing Thioredoxin-Interacting Protein (TXNIP) and Suppressing ER Stress in C2C12 Myotubes. Front Pharmacol. 2018;9:1180.

25. Sano S, Izumi Y, Yamaguchi T, Yamazaki T, Tanaka M, Shiota M, Osada-Oka M, Nakamura Y, Wei M, Wanibuchi H, Iwao H, Yoshiyama M. Lipid synthesis is promoted by hypoxic adipocyte-derived exosomes in 3T3-L1 cells. Biochem Biophys Res Commun. 2014;445(2):327-33.

26. Zhang H, Liu J, Qu D, Wang L, Luo JY, Lau CW, Liu P, Gao Z, Tipoe GL, Lee HK, Ng CF, Ma RC, Yao X, Huang Y. Inhibition of miR-200c Restores Endothelial Function in Diabetic Mice Through Suppression of COX-2. Diabetes. 2016;65(5):1196-207.

27. Higuchi C, Nakatsuka A, Eguchi J, Teshigawara S, Kanzaki M, Katayama A, Yamaguchi S, Takahashi N, Murakami K, Ogawa D, Sasaki S, Makino H, Wada J. Identification of circulating miR-101, miR-375 and miR-802 as biomarkers for type 2 diabetes. Metabolism. 2015;64(4):489-97.

28. Lin N, Niu Y, Zhang W, Li X, Yang Z, Su Q. microRNA-802 is involved in palmitate-
induced damage to pancreatic β cells through repression of sirtuin 6. Int J Clin Exp Pathol. 2017;10(11):11300-7.

29. Wang C, Mao X, Wang L, Liu M, Wetzel MD, Guan KL, Dong LQ, Liu F. Adiponectin sensitizes insulin signaling by reducing p70 S6 kinase-mediated serine phosphorylation of IRS-1. J Biol Chem. 2007;282(11):7991-6.

30. Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, Hatazaki M, Matsuoka TA, Ozawa K, Ogawa S, Hori M, Yamasaki Y, Matsuhisa M. Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. J Biol Chem. 2005;280(1):847-51.

31. Nishikawa T, Araki E. Impact of mitochondrial ROS production in the pathogenesis of diabetes mellitus and its complications. Antioxid Redox Signal. 2007;9(3):343-53.

32. Fedorova M, Bollineni RC, Hoffmann R. Protein carbonylation as a major hallmark of oxidative damage: update of analytical strategies. Mass Spectrom Rev. 2014;33(2):79-97.

33. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta. 2003;329(1-2):23-38.

34. Selthofer-Relatić K, Kibel A, Delić-Brkljačić D, Bošnjak I. Cardiac Obesity and Cardiac Cachexia: Is There a Pathophysiological Link? J Obes. 2019;2019:9854085.

35. González N, Moreno-Villegas Z, González-Bris A, Egido J, Lorenzo Ó. Regulation of visceral and epicardial adipose tissue for preventing cardiovascular injuries associated to obesity and diabetes. Cardiovasc Diabetol. 2017;16(1):44.

36. Gan L, Xie D, Liu J, Lau WB, Christopher TA, Lopez B, Zhang L, Gao E, Koch W, Ma XL, Wang Y. Small Extracellular Microvesicles Mediated Pathological Communications between Dysfunctional Adipocytes and Cardiomyocytes as a Novel Mechanisms Exacerbating Ischemia/Reperfusion Injury in Diabetic Mice. Circulation. 2020 Jan 10. doi: 10.1161/CIRCULATIONAHA.119.042640. [Epub ahead of print].
37. Yu Y, Du H, Wei S, Feng L, Li J, Yao F, Zhang M, Hatch GM, Chen L. Adipocyte-Derived Exosomal MiR-27a Induces Insulin Resistance in Skeletal Muscle Through Repression of PPARγ. Theranostics. 2018;8(8):2171-88.

38. Ni M, Zhao Y, Zhang WJ, Jiang YJ, Fu H, Huang F, Li DJ, Shen FM. microRNA-802 accelerates hepatocellular carcinoma growth by targeting RUNX3. J Cell Physiol. 2020 Jan 31. doi: 10.1002/jcp.29611. [Epub ahead of print].

39. You Q, Wang J, Jia D, Jiang Y, Chang Y, Li W. MiR-802 alleviates lipopolysaccharide-induced acute lung injury by targeting Peli2. Inflamm Res. 2020;69(1):75-85.

40. Sansom SE, Nuovo GJ, Martin MM, Kotha SR, Parinandi NL, Elton TS. miR-802 regulates human angiotensin II type 1 receptor expression in intestinal epithelial C2BBe1 cells. Am J Physiol Gastrointest Liver Physiol. 2010;299(3):G632-42.

41. Kornfeld JW, Baitzel C, Könner AC, Nicholls HT, Vogt MC, Herrmanns K, Scheja L, Haumaitre C, Wolf AM, Knippschild U, Seibler J, Cereghini S, Heeren J, Stoffel M, Brüning JC. Obesity-induced overexpression of miR-802 impairs glucose metabolism through silencing of Hnf1b. Nature. 2013;494(7435):111-5.

42. Sun D, Chen J, Wu W, Tang J, Luo L, Zhang K, Jin L, Lin S, Gao Y, Yan X, Zhang C. MiR-802 causes nephropathy by suppressing NF-κB-repressing factor in obese mice and human. J Cell Mol Med. 2019;23(4):2863-2871.

43. Gupta S, Knowlton AA. HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. Am J Physiol Heart Circ Physiol. 2007;292(6):H3052-6.

44. Heiserman JP, Chen L, Kim BS, Kim SC, Tran AL, Siebenborn N, Knowlton AA. TLR4 mutation and HSP60-induced cell death in adult mouse cardiac myocytes. Cell Stress Chaperones. 2015;20(3):527-35.

45. Mandal K, Jahangiri M, Xu Q. Autoimmunity to heat shock proteins in atherosclerosis. Autoimmun Rev. 2004;3(2):31-7.
46. Wick G, Knoflach M, Xu Q. Autoimmune and inflammatory mechanisms in atherosclerosis. Annu Rev Immunol. 2004;22:361-403.

47. Lindsberg PJ, Grau AJ. Inflammation and infections as risk factors for ischemic stroke. Stroke. 2003;34(10):2518-32.

48. Chen TH, Liu SW, Chen MR, Cho KH, Chen TY, Chu PH, Kao YY, Hsu CH, Lin KM. Neonatal Death and Heart Failure in Mouse with Transgenic HSP60 Expression. Biomed Res Int. 2015;2015:539805.

49. Shan YX, Yang TL, Mestril R, Wang PH. Hsp10 and Hsp60 suppress ubiquitination of insulin-like growth factor-1 receptor and augment insulinlike growth factor-1 receptor signaling in cardiac muscle: Implications on decreased myocardial protection in diabetic cardiomyopathy. J Biol Chem 2003;278:45492-8.

50. Lin L, Kim SC, Wang Y, Gupta S, Davis B, Simon SI, Torre-Amione G, Knowlton AA. HSP60 in heart failure: abnormal distribution and role in cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol. 2007;293(4):H2238-47.

51. Fan F, Duan Y, Yang F, Trexler C, Wang H, Huang L, Li Y, Tang H, Wang G, Fang X, Liu J, Jia N, Chen J, Ouyang K. Deletion of heat shock protein 60 in adult mouse cardiomyocytes perturbs mitochondrial protein homeostasis and causes heart failure. Cell Death Differ. 2020;27(2):587-600.

52. Lau S, Patnaik N, Sayen MR, Mestril R. Simultaneous overexpression of two stress proteins in rat cardiomyocytes and myogenic cells confers protection against ischemia-induced injury. Circulation. 1997;96:2287-94.

53. Lin KM, Lin B, Lian IY, Mestril R, Scheffler IE, Dillmann WH. Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemiareoxygenation. Circulation. 2001;103:1787-92.
54. Achari AE, Jain SK. Adiponectin, a Therapeutic Target for Obesity, Diabetes, and Endothelial Dysfunction. Int J Mol Sci. 2017;18(6). pii: E1321.

55. Shibata R, Ouchi N, Ito M, Kihara S, Shiojima I, Pimentel DR, Kumada M, Sato K, Schiekofer S, Ohashi K, Funahashi T, Colucci WS, Walsh K. Adiponectin-mediated modulation of hypertrophic signals in the heart. Nat Med. 2004;10(12):1384-9.

56. Frankenberg ADV, Reis AF, Gerchman F. Relationships between adiponectin levels, the metabolic syndrome, and type 2 diabetes: a literature review. Arch Endocrinol Metab. 2017;61(6):614-22.

Supplemental Information

Additional file 1: Figure S1. Inhibition of miR-802-5p in 3T3-L1 adipocytes attenuated exosome-induced insulin resistance in NRVMs. 3T3-L1 adipocytes were transfected with miR-802-5p inhibitor and then treated with 0.5 mmo/L PA to induce hypertrophic adipocytes. Exosomes were purified from the conditioned medium. NRVMs were starved serum for 6 h and then treated with exosomes for another 18 h, followed by stimulation with or without 100 nmol/L insulin for 10 min. (a, b) Effects of exosomes on Akt T308 phosphorylation. (c) Effects of exosomes on 2-DG uptake. n=4. ** p<0.01, *** p<0.001 vs the indicated group (one-way ANOVA). nc-Exo: exosomes purified from normal control 3T3-L1 adipocytes; h-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes; miR-inh-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes with miR-802-5p inhibition; INS: insulin.

Additional file 2: Figure S2. Loss of HSP60 mitigated insulin-sensitizing action of adiponectin in NRVMs. NRVMs transfected with HSP60 siRNA or it control sequence were starved serum for 18 h and then treated with 2 μg/mL globular adiponectin for 60 min, followed by stimulation with 100 nmol/L insulin for 10 min. (a, b) Effects of HSP60 knockdown on Akt T308 phosphorylation. (c) Effects of HSP60 knockdown on 2-DG uptake. n=4. ** p<0.01, *** p<0.001 vs the indicated group (one-way ANOVA). INS: insulin.

Additional file 3: Figure S3. Inhibition of miR-802-5p in 3T3-L1 adipocytes attenuated
**exosome-induced UPR and ROS in NRVMs.** 3T3-L1 adipocytes were transfected with miR-802-5p inhibitor and then treated with 0.5 mmol/L PA to induce hypertrophic adipocytes. Exosomes were purified from the conditioned medium. NRVMs were treated with exosomes for another 18 h under serum starvation condition. (a-c) Effects of exosomes on the markers of ER stress and the UPR. (d) Effects of exosomes on intracellular ROS formation. (e) Effects of exosomes on carbonylated proteins. n=4. **p<0.01, ***p<0.001 vs the indicated group (one-way ANOVA). nc-Exo: exosomes purified from normal control 3T3-L1 adipocytes; h-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes; miR-inh-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes with miR-802-5p inhibition.

**Figures**

**Figure 1**

Hypertrophic adipocyte-derived exosome induced insulin resistance in NRVMs. NRVMs were starved serum for 6 h and then treated with exosomes for another 18 h, followed by stimulation with or without 100 nmol/L insulin for 10 min. (a, b) Effect of exosomes on Akt T308 phosphorylation. (c) Effect of exosomes on 2-DG uptake. n=4. **p<0.01, ***p<0.001 vs the indicated group (one-way ANOVA). nc-Exo: exosomes purified from normal control 3T3-L1 adipocytes; h-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes; CytoD: cytochalasin D; INS: insulin.
Exosomal miR-802-5p contributed to insulin resistance in NRVMs. (a) quantification of miR-802-5p concentration in exosomes. NRVMs transfected with miR-802-5p mimic, inhibitor, or their paired control were starved serum for 18 h and then stimulated with or without 100 nmol/L insulin for 10 min. (b, c) Effects of miR-802-5p mimic on Akt T308 phosphorylation. (d) Effects of miR-802-5p mimic on 2-DG uptake. (e, f) Effects of miR-802-5p inhibitor on Akt T308 phosphorylation. (g) Effects of miR-802-5p inhibitor on 2-DG uptake. n=4. *** p<0.001 vs the indicated group (one-way ANOVA). nc-Exo: exosomes purified from normal control 3T3-L1 adipocytes; h-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes; inh: inhibitor; INS: insulin.
Figure 3

Mir-802-5p impaired insulin signaling by targeting HSP60. (a) The predicted miR-802-5p binding site in the 3’UTR of the Hspd1 gene from TargetScan online software. (b) Luciferase reporter test of miR-802-5p and Hspd1. (c, d) Effects of exosomes on HSP60 protein levels. (e, f) Effects of exosomes with miR-802-5p inhibition on HSP60 protein levels. (g, h) Effects of miR-802-5p mimic on HSP60 protein levels. n=4. ** p<0.01, *** p<0.001 vs the indicated group (one-way ANOVA). nc-Exo: exosomes purified from normal control 3T3-L1 adipocytes; h-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes; miR-inh-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes with miR-802-5p inhibition.
Loss of HSP60 induced insulin resistance in NRVMs. NRVMs transfected with HSP60 siRNA or its control sequence were starved serum for 18 h and then stimulated with 100 nmol/L insulin for 10 min. (a, b) Effects of HSP60 knockdown on Akt T308 phosphorylation. (c) Effects of HSP60 knockdown on 2-DG uptake. n=4. ** p<0.01, *** p<0.001 vs the indicated group (one-way ANOVA). INS: insulin.
HSP60 depletion resulted in the UPR and ROS in NRVMs. NRVMs transfected with HSP60 siRNA or its control sequence were starved serum for 18 h. (a-c) Effects of HSP60 knockdown on the markers of ER stress and the UPR. (d) Effects of HSP60 knockdown on intracellular ROS formation. (e) Effects of HSP60 knockdown on carbonylated proteins. (f, g) Effects of HSP60 knockdown on phosphorylation of JNK and IRS-1 S307. n=4. * p<0.05, ** p<0.01, *** p<0.001 vs the indicated group (one-way ANOVA).
Exosomal miR-802-5p induced the UPR and ROS in NRVMs. NRVMs were treated with exosomes in presence or absence of CytoD for 18 h under serum starvation condition. (a-c) Effects of exosomes on the markers of ER stress and the UPR. (d) Effects of exosomes on intracellular ROS formation. (e) Effects of exosomes on carbonylated proteins. NRVMs transfected with miR-802-5p mimic and its control sequence were starved serum for 18 h. (f-h) Effects of miR-802-5p mimic on the markers of ER stress and the UPR. (i) Effects of miR-802-5p mimic on intracellular ROS formation. (j) Effects of miR-802-5p mimic on carbonylated proteins. n=4. *** p<0.01, *** p<0.001 vs the indicated group (one-way ANOVA). nc-Exo: exosomes purified from normal control 3T3-L1 adipocytes; h-Exo: exosomes purified from hypertrophic 3T3-L1 adipocytes; CytoD: cytochalasin D.

Hypertrophic Adipocytes
Figure 7

Schematic diagram of exosomal miR-802-5p on insulin signaling. Hypertrophic adipocytes accelerate the releases of exosomal miR-802-5p leading to reduction of HSP60 proteins in NRVMs. In addition, HSP60 deficiency results in the UPR and ROS, both of them contributes to insulin resistance in NRVMs.

Supplementary Files
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