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

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Research

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Abstract

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. We found that 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. In summary, 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% CO_2 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'-GAGAGGTGTGATGTTGGCTGTTGAT-3' and 5'- GAGTGTGGTAGGGTTTGTCTGAGAT-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 our 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) (Figs. 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 (Fig. 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 (Figs. 3c and d) and miR-802-5p (Figs. 3e and f). In addition, miR-802-5p mimic significantly downregulated HSP60 protein levels in NRVMs (Figs. 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 (Figs. 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 (Figs. S2a and b) and glucose uptake (Fig. 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 (Figs. 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 (Figs. 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 (Figs. 6a-c), ROS formation (Fig. 6d), and protein carbonylation (Fig. 6e). Similar observations were made in NRVMs treated with miR-802-5p mimic (Figs. 6f-j). These h-Exo-induced changes were suppressed by endocytosis inhibitor CytoD (Figs. 6a-e). In addition, miR-inh-Exo-exposed NRVMs displayed obvious reductions in PERK phosphorylation and CHOP protein expression (Figs. S3a-c), ROS formation (Fig. S3d), and protein carbonylation (Fig. S3e), 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 (Figs. 1 and 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 (Figs. 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 (Fig. 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 (Figs. 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

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials:

The datasets used and/or analyzed in this study are available from the corresponding author upon reasonable request.

Competing interests:

The authors declare that they have no competing interests.

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Author'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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Supplemental Figure Legends

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

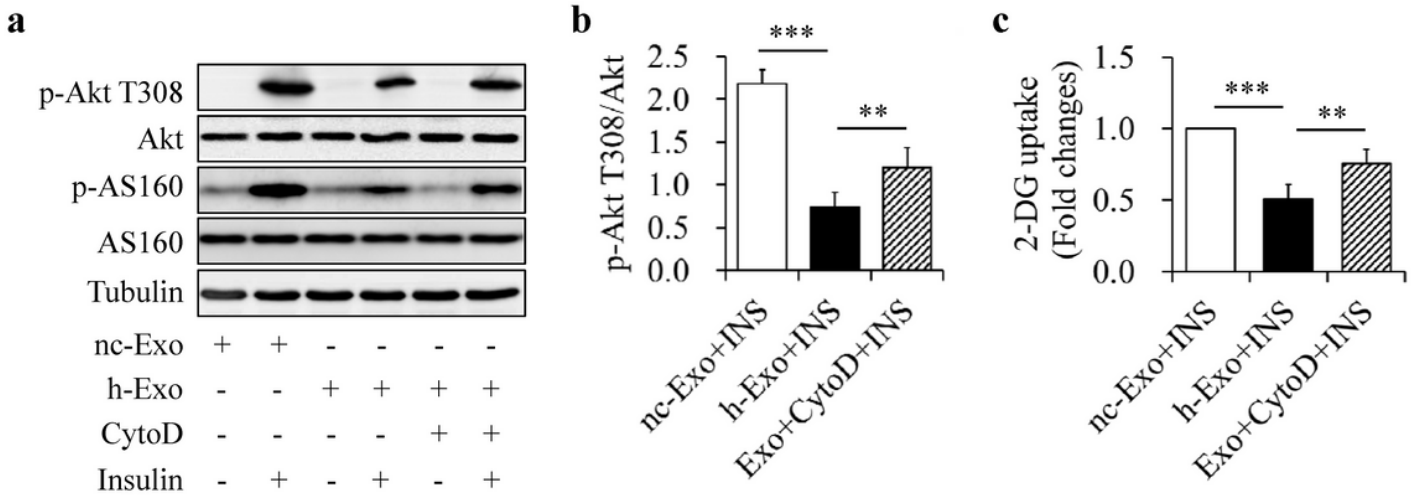

Figure 1

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.

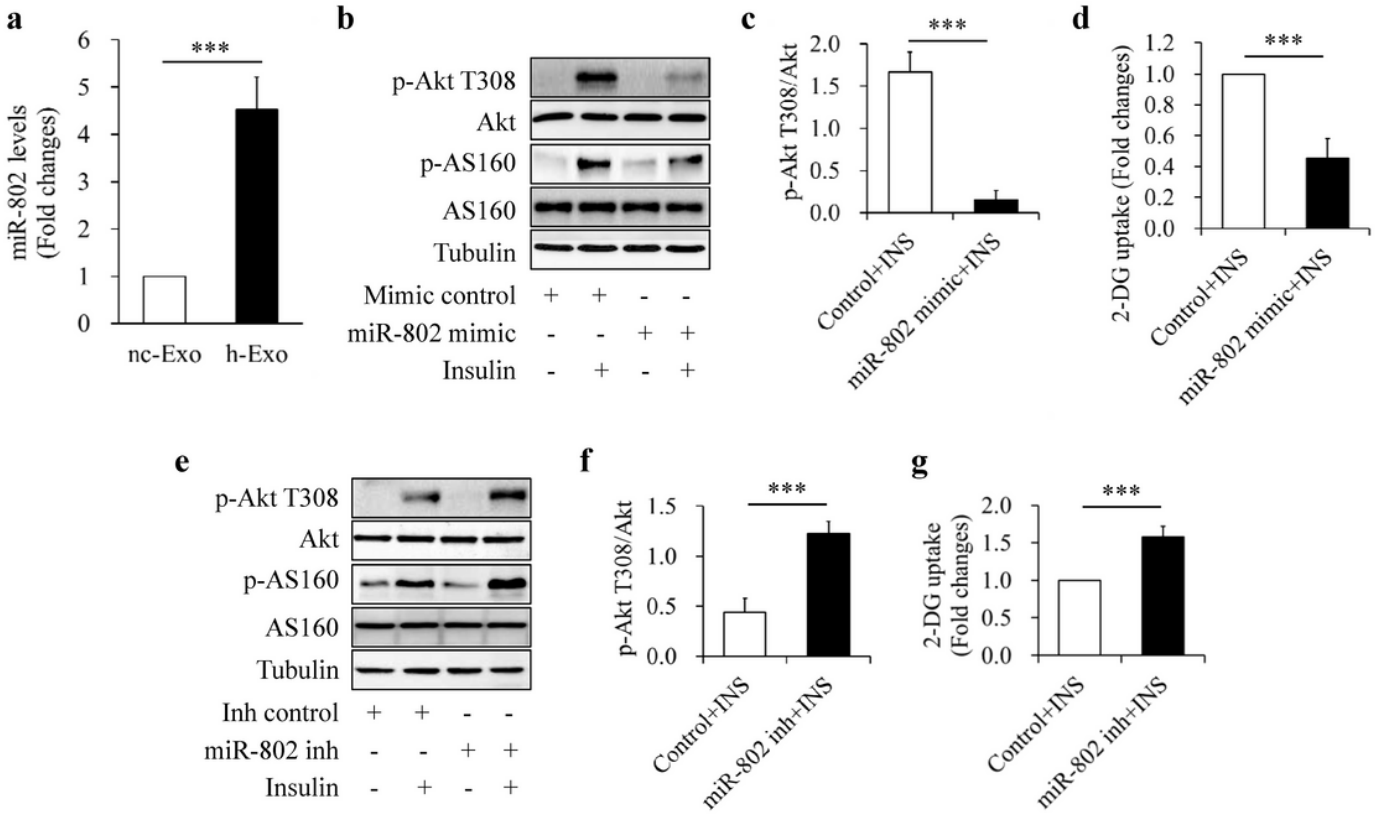

Figure 3

Exosomal miR-802-5p contributed to insulin resistance in NRVMs. (a) quantification of miR-802-5p concentration in exosomes. NRMVs 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.

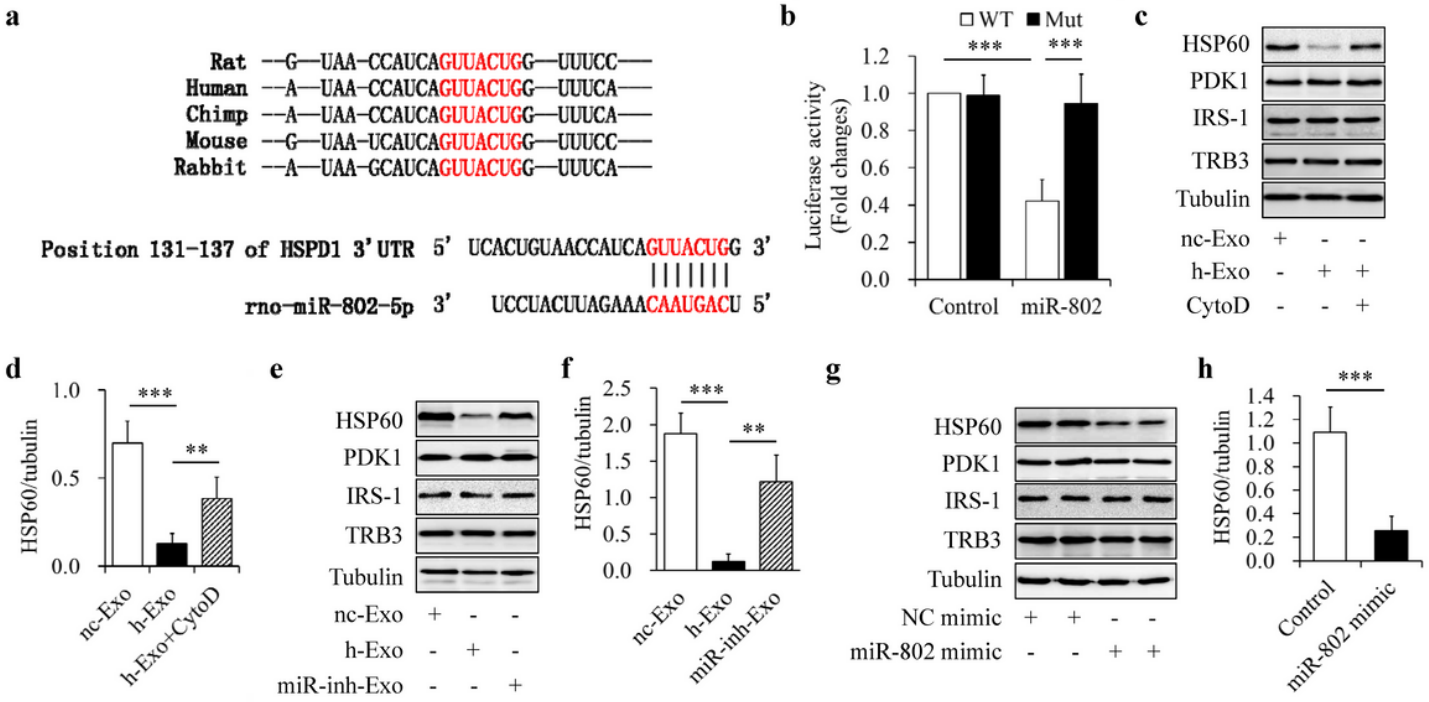

Figure 5

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.

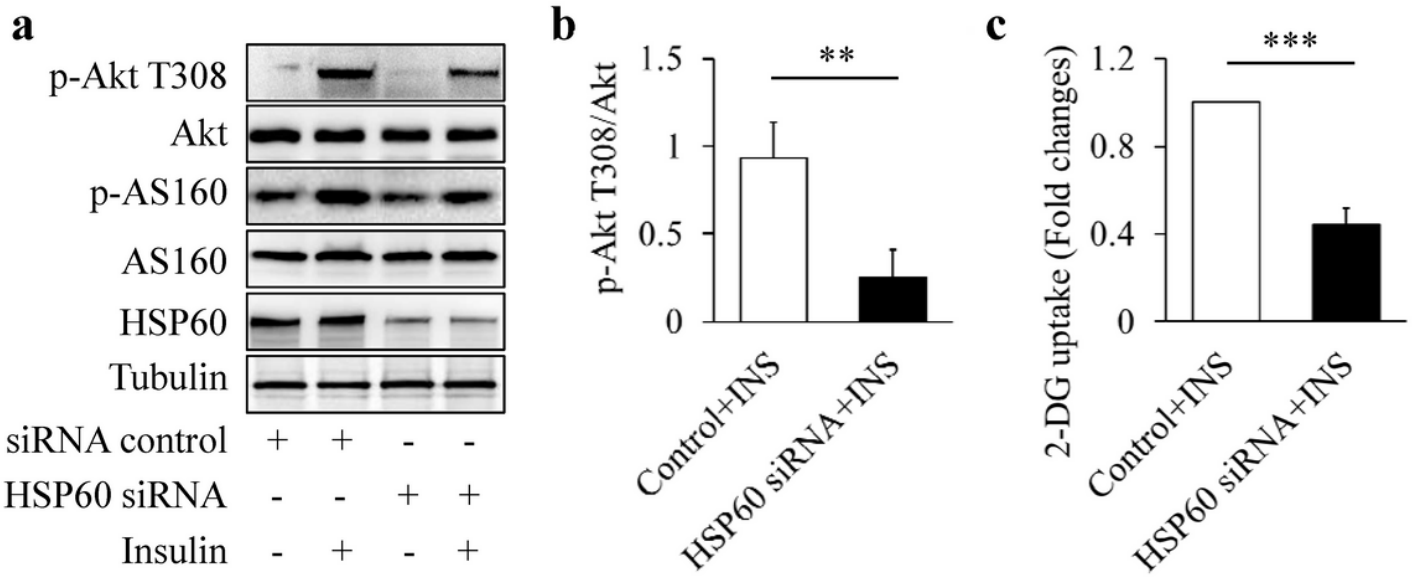

Figure 7

Loss of HSP60 induced insulin resistance in NRVMs. NRVMs transfected with HSP60 siRNA or it 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.

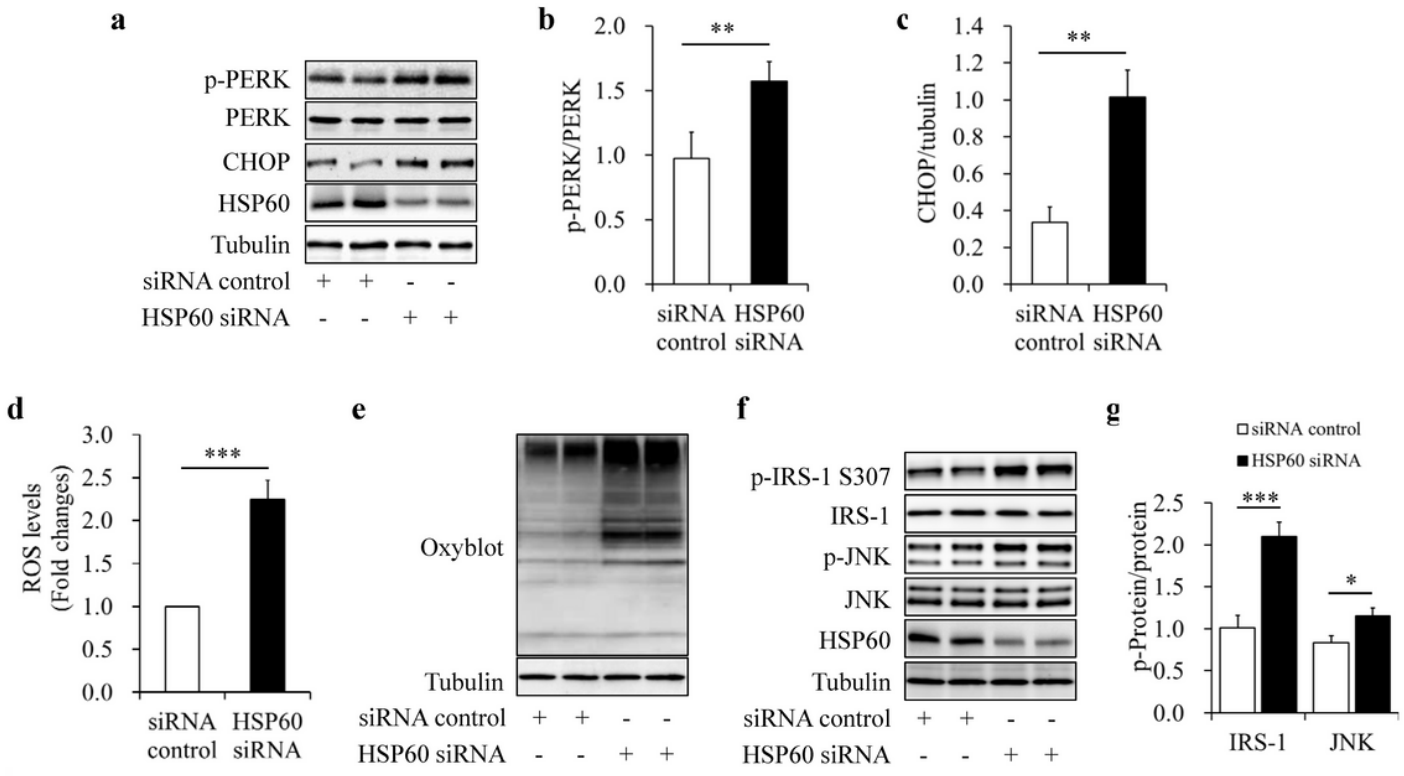

Figure 9

HSP60 depletion resulted in the UPR and ROS in NRVMs. NRVMs transfected with HSP60 siRNA or it 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).

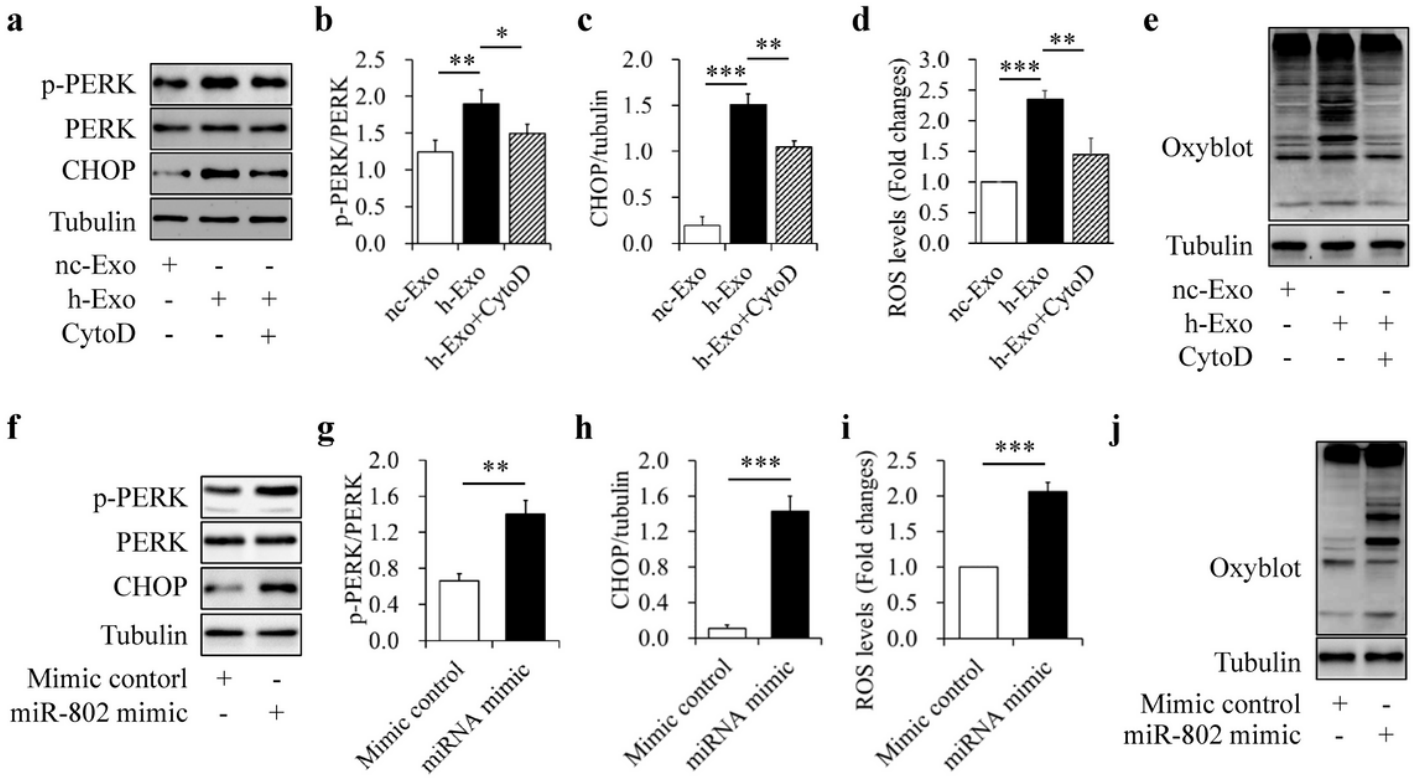

Figure 11

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.

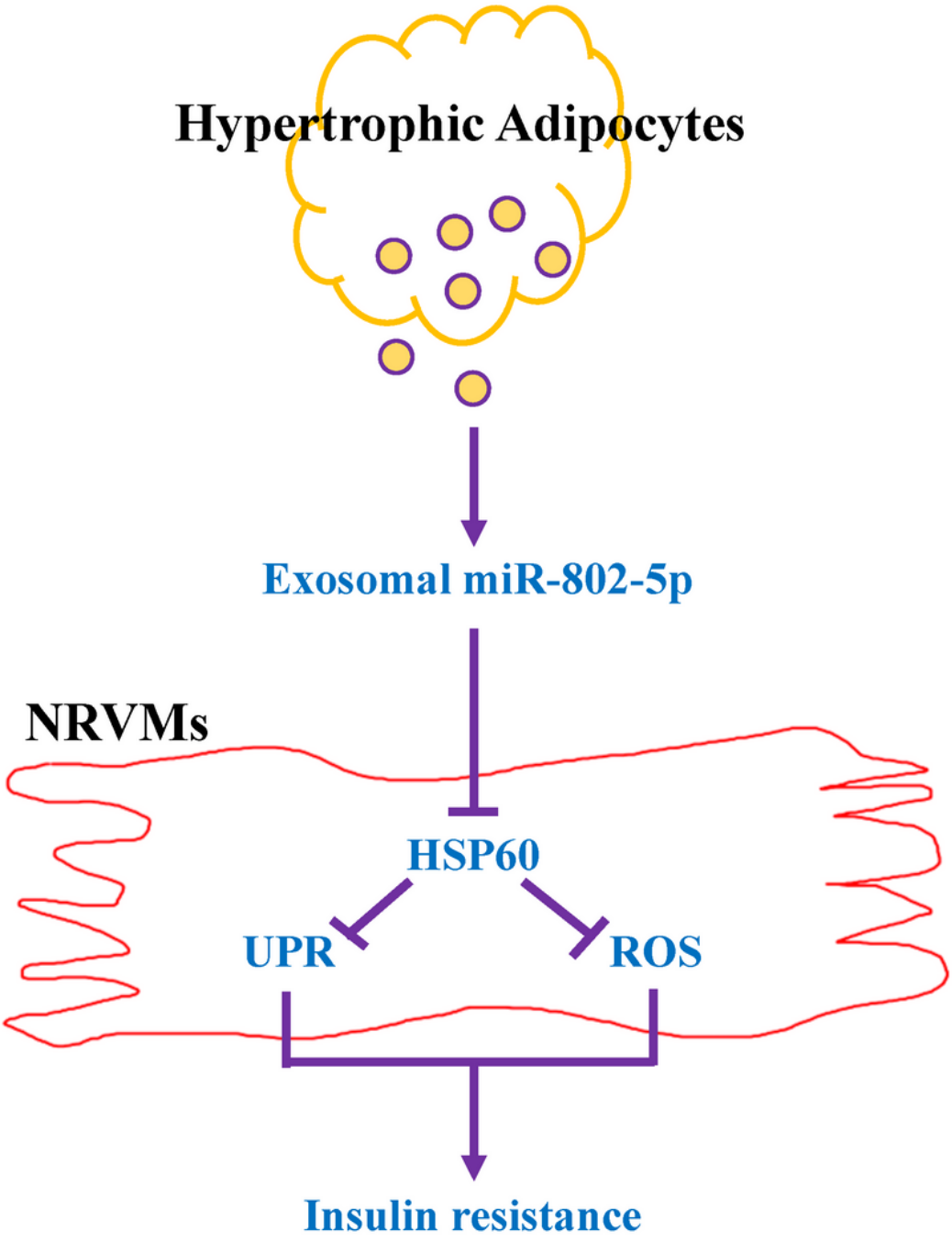

Figure 13

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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