Extracellular vesicle microRNA cargoes from intermittent hypoxia-exposed cardiomyocytes and their effect on endothelium

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Research

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Abstract

Background

Intermittent hypoxia (IH), as the crucial pathophysiological feature of obstructive sleep apnea syndrome (OSAS), is an independent risk factor initiating the progression of cardiovascular complications. However, how IH cause cardiovascular injury and initiate inter-organ communication remains unclear. Extracellular vesicle (EV) are reported to be involved in cell-to-cell and organ-to-organ communications through selectively carrying RNA and protein cargos from donor cells and delivering them to recipient cells. By utilizing an unbiased miRNA microarray approach, current study attempted to determine whether IH may alter miRNA profiles in cardiomyocyte-derived extracellular vesicles and whether EVs from IH-treated cardiomyocytes could affect endothelial function.

Methods

EVs were isolated from culture medium of normoxia- or intermittent hypoxia-treated C57BL/6 mouse primary cardiomyocytes. miRNA array assay was used to identify myocardial EV miRNA. The expression of target miRNAs was validated by qPCR and the enriched function of the target miRNAs were predicted by bioinformatics and western blotting analysis. Moreover, vascular functional study validated whether EVs from IH-exposed cardiomyocytes could influence endothelial function.

Results

63 differentially expressed miRNAs, including 32 up-regulated and 31 down-regulated miRNAs were identified in EVs from IH-exposed cardiomyocytes. Among them, 16 miRNAs with homologous sequence in mouse and human were selected and verified by qPCR assay and 11 miRNAs were proved with the same tendency as miRNA array identified. KEGG pathway and western blotting analysis showed the main enriched pathway of differentially expressed miRNA was associated with PI3K/Akt signaling pathway. Of note, EVs from IH-exposed cardiomyocytes dramatically impaired endothelium-dependent relaxation and inhibited Akt/eNOS pathway in endothelial cells.

Conclusions

This study provides the first evidence that IH causes significant alteration in myocardial EV miRNA composition, which may contribute to IH or OSAS-triggered cardiovascular injury and organ-to-organ communication.

Background
Obstructive sleep apnea syndrome (OSAS) is an independent risk factor associated with many clinical complications (i.e., hypertension, stroke and coronary heart disease) [1]. Intermittent hypoxia (IH) which commonly seen as the key property of OSAS is considered as the major pathological origin of OSAS-associated cardiovascular diseases. Previous findings suggest that IH resulted in cardiomyocytes apoptosis because of insufficient oxygen supply[2] or through the mediation of miR-146a-5p[3]. Additionally, IH exposure caused earlier and greater inflammatory response in cardiomyocytes as evidenced by increased lactate dehydrogenase and proinflammatory cytokine (IL-1β, IL-6, IL-8, and macrophage migration inhibitory factor) [4]. Recent work of our group proved that myocardial infarction plus IH treatment exacerbates cardiac remodeling via miR-214-3p/CTRP9 pathway[5]. Although previous researches have been devoted to seeking for molecular targets to alleviate IH-related cardiac injury, whereas more novel biomarkers are still needed to be discovered due to the insufficient findings as yet.

Extracellular vesicles (EVs) are nano-scale bilayer membrane microvesicles produced by most of cells. The components of EVs closely reflect the physiological and pathological status of their tissue origins and thus are considered as desirable targets for clinical diagnosis and therapeutics. Meanwhile, EVs participate in extensive physiological and pathological processes by transferring complex cargoes including proteins, lipids, and nucleic acids from donor cells to recipient cells[6]. Lots of studies have shown that EV miRNAs are involved in cardiovascular disease[7]. Cardiac fibroblast EV-miR-423-3p was reported participated in cardioprotective effects through targeting the downstream effector Rap-2c during the acute phase of ischemia–reperfusion injury[8]. While cardiomyocyte-derived EV miR-208a participated in cardiac fibrosis via promoting fibroblast proliferation and differentiation into myofibroblasts[9]. The decreased EV microRNA-21-5p derived from heart failure patients impaired cardiac regenerative potential by inhibiting angiogenesis and cardiomyocyte survival[10]. And the enriched miR-130b-3p in dysfunctional adipocyte-derived EV was found by our team mediating diabetes-related cardiac injury through suppression of cardioprotective molecules expression in cardiomyocytes[11]. Therefore, systematical and precise dissection of the myocardium-derived EV moleculars under IH conditions is so important as that will help to determine the main mechanism of OSAS or IH-associated cardiovascular diseases, and subsequently aid to develop more effective and precise diagnosis and treatment in clinical practice. However, the disparity of miRNA components in EVs delivered from normoxia-and IH-treated cardiomyocytes is not yet clear.

This study provides the first evidence that IH causes significant alteration in myocardial EV miRNA composition and reveals the adverse effect and the possible mechanism of EVs from IH-treated cardiomyocytes on endothelial function, which will help to understand the mechanistic underpinnings behind the pathology of OSAS and IH and their related cardiovascular diseases.

**Methods**

**Cell culture**
All animal handling complied with the standard animal welfare regulations of Capital Medical University. The Animal Subjects Committee of Capital Medical University approved the animal study protocol. C57BL/6 mouse primary cardiomyocytes were isolated as described previously with minor modifications[12]. Briefly, adult male C57BL/6 mice (8-12 weeks) were subjected to general anesthesia (2% isoflurane). Hearts were harvested and perfused retrogradely via aortic cannulation at 37°C for 5 minutes with a constant flow of Perfusion Buffer (116 mM NaCl, 5.4 mM KCl, 6.7 mM MgCl₂, 12 mM glucose, 2 mM glutamine, 3.5 mM NaHCO₃, 1.5 mM KH₂PO₄, 1.0 mM NaH₂PO₄, 21 mM HEPES) 4.5 mL/min in a Langendorf apparatus (WPI, United States). Then hearts were followed by perfused again in digestion solution supplemented with 0.6 mg/ml collagenase II and 15 mM CaCl₂ at 37°C for 10 minutes. After that, hearts were quickly triturated the ventricular tissue with fine forceps, so that most pieces were smaller than 1 mm³. Next the cell suspension was transferred to a 15 mL conical, allowed to settle by gravity for 15 min and carefully removed the supernatant with a transfer pipette to cell culture dishes until only 50 - 100 μL of solution remained above the tissue pieces. Then the cell culture dish was moved promptly to a 37 °C incubator with 5% CO₂ and 95% humidity.

HUVECs were purchased from AllCells (Shanghai, China) and cultured with a special medium ECM (ScienCell, San Diego, CA, USA). The 8-10 generations were used for our study and cells were collected for protein detection after treatment with EVs for 24 h or 48h respectively.

**Intermittent hypoxia treatment**

Intermittent hypoxia (IH) condition was obtained by cycles of 5%-21% O₂ per hour with a BioSpherix-OxyCycler C42 system (BioSpherix, Redfield, NY, USA). Mouse primary cardiomyocytes were subjected to IH or normoxia (Nor) treatment (continuous 21% O₂ as control) for 48h with EV-free medium (SBI, USA) incubation. Then the conditioned medium was collected.

**EV purification**

EVs were isolated from cardiomyocyte culture media according to the method previously described with simple modifications[13]. Briefly, the culture medium of cardiomyocytes exposed to IH or Nor status was collected and centrifuged progressively at 300 × g for 15 min, 2,000 × g for 15 min, 12,000 × g for 80 min to remove cardiomyocytes and cell debris. Then, the supernatant was passed through a 0.1μm microporous membrane filter (Millipore, MA, USA) and ultracentrifuged at 110,000 × g for 120 min (Beckman Coulter, CA, USA). The EV pellets were washed with PBS to exclude contaminating proteins followed by a second ultracentrifugation at 110,000 × g for 120 min. All the centrifugation steps were performed at 4°C. EVs purified from 50mL culture medium of C57BL/6 mouse primary cardiomyocytes (from 5 mice) were collected and frozen at -80°C as one sample for miRNA microarray chip assay.

**EVs identification**

The characterization of EVs was confirmed by measuring the expression of EV-specific markers, such as CD63, CD81, TSG101, Alix and calnexin via Western blotting analysis. The particle size and number were
detected by NanoSight analysis (NS300, Malvern Instruments) and the EV morphology was visualized by transmission electron microscope (TEM, H-600, Hitachi, 

**Western blotting and silver staining**

Protein was extracted from EVs using a protein extraction kit (BC3710, Solarbio, Beijing, China) and protein concentration was quantified using a BCA assay (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Protein (5 µg) was separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (EMD Millipore, USA). After blocked with 5% nonfat milk, the membranes were incubated with primary antibodies overnight at 4°C. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; K5007; Dako, USA) for 1 h at room temperature. The protein bands were visualized using the SuperSignal chemiluminescent detection module (34080; Pierce) and images were collected using a ChemiDoc™ Touch (Bio-Rad, USA). Silver staining was performed with the Silver Staining Kit (Invitrogen, USA) following the manufacturer's recommendations. The primary antibody information can be found in Table S2.

**Transmission electron microscopy**

The morphology of EVs was observed using transmission electron microscopy (TEM). Electron microscope analysis of whole-mounted EVs was carried out according to the reported protocol [13]. Briefly, the EV pellets were fixed with 2% PFA and deposited onto EM grids. The grids were separately transferred into 1% glutaraldehyde and methyl cellulose-UA for 10 mins. After air dry, the grid was viewed under FEI Tecnai electron microscope at 80 kV.

**miRNA library preparation, microarray chip Assay, and data analysis**

EV miRNAs were extracted by using the miRNA isolation Kit (catalog number AM1561, Thermo Fisher Scientific, USA) according to manufacturer's protocol. MicroRNA array analysis was undertaken by Oebiotech Co., Ltd. (Shanghai, China). Briefly, 100ng total miRNA per sample served as an input material for the small RNA library preparation. The libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, E7300L). The library quality was assessed on the Agilent Bioanalyzer 2100 system using miRNA High Sensitivity Chips. The chip was sequenced on an Agilent Mouse miRNA 21.0 platform. Known miRNAs were identified by comparison to miRBase 21.0 database (http://www.mirbase.org/). The p value < 0.05 and |log₂ (fold change)| > 1 was set as the threshold for significantly differential expression by default. Differential miRNAs were exhibited by volcano plot and hierarchical clustering heatmap.

**Real-time quantitative PCR**

Reverse transcription and qPCR were carried out by using Hairpin-it MicroRNA RT-PCR Quantitation Kit (catalog number E22001; GenePharma, China) with 2 ng EV miRNA according to the manufacturer's procedures. The miRNA stem-loop PCR primers supplied by Hairpin-it MicroRNA RT-PCR Quantitation Kit
were shown in Table S3. The expression of miRNAs (let-7j, miR-23a-3p, miR-181b-5p, miR-29a-3p, miR-214-5p, miR-125b-5p, miR-448-5p, miR-494-3p, miR-1a-3p, miR-16-2-3p, miR-504-3p, miR-30c-5p, miR-129b-5p, miR-133b-3p, miR-101a-5p and miR-22-3p) was determined using the 7500 System (Applied Biosystems, USA). The expression levels of all detected miRNAs was presented in terms of fold change normalized to U6 expression using the formula $2^{-\Delta \Delta C_t}$. Predicted targets of significant up- or down-regulated miRNAs were computationally retrieved from the TargetScan (http://www.targetscan.org/vert_71/), microRNA.org (http://www.microrna.org/) and PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_dyn_data.html) databases. The common protein targets of differentially expressed miRNAs predicted by three databases were analyzed by DAVID bioinformatic tool (https://david.ncifcrf.gov/) in order to perform functional annotation (GO) and biological pathway enrichment (KEGG) analysis.

**Vascular functional study**

Adult male C57BL/6 mice (8-12 weeks) were anaesthetized with isoflurane and aortas were dissected out for functional assay. Aortic segments (3 mm in length) were suspended in wire myograph (Danish Myo Technology, Aarhus N, Denmark) to record changes in isometric force. After the activation of phenylephrine (Phe, 1 μM) in the organ bath, the vascular tone of REV-treated aorta rings raised from basic 3 mN to 5-7 mN. Then, endothelium-dependent vasodilatation relaxation was assessed by measuring the dilatory responses to cumulative concentrations of acetylcholine (ACh). In addition, endothelium-independent relaxation was assessed by measuring the dilatory responses to cumulative concentrations of sodium nitroprusside (SNP) after aorta rings were contracted by NG-nitro-L-arginine methyl ester (L-NAME, 100 μM).

**Statistical analysis**

Data are shown as mean ± SD for numerical variables. Comparisons were made using the Student's t-test, as appropriate. For all statistical tests, p values less than 0.05 were considered statistically significant. All statistical analyses were performed with Graphpad Prism 7.

**Results**

**Characteristics of cardiomyocyte extracellular vesicles**

Myocardium-derived EVs were isolated by a combination of ultracentrifugation and filtration. EVs were visualized by transmission electron microscopy after negative staining for morphology and size determination. Results showed that the cardiomyocyte EVs exhibited a near-spherical shape and typical disk-shaped morphology (Fig. 1A). Western blotting analysis indicated that the purified EVs contained cardiomyocyte specificity marker α-sarcomeric actin (α-SA) and the typical exosomal markers CD63, CD81, Alix, Tsg101 without endoplasmic reticulum protein Calnexin (Fig. 1B and Additional file2: Supplemental Table 2). The size distribution of EVs detected by Nanosight NS300 (Malvern, UK) presented that the average diameters of EVs were approximately 121.4±28.9 nm (Nor) and
135.34±17.23nm (IH). The diameters of around 60% EVs were between 40 and 80 nm and more than 80% EVs were less than 200 nm (Fig. 1C). Meanwhile, the number of EVs from IH-treated cardiomyocytes was observed significantly higher than its counterpart (Fig. 1D).

**Microarray chip assay and bioinformatic analysis of EV miRNAs from Nor- or IH-treated cardiomyocytes**

To investigate whether IH affects myocardium EV miRNA composition, miRNA microarray analysis was performed. Three biological replicates were performed to obtain reliable results (Fig. 2A). We quantified a total of 1,944 miRNAs from myocardial EVs in the three replicates by comparing to miRBase 21.0 database (http://www.mirbase.org/). To reveal the functional relevance of myocardial EVs under IH status, we mainly focused on the analysis of the differentially expressed miRNAs identified in EVs from IH- and Nor-treated cardiomyocytes. The differentially expressed miRNAs were defined as miRNA with 2-fold cutoff and p value less than 0.05. Using these criteria, 63 differentially expressed microRNAs were identified in IH-treated myocardial EVs including 32 up-regulated microRNAs and 31 down-regulated miRNAs (Fig. 2B and Additional file1: supplemental table 1). The target genes of differentially expressed myocardial EV miRNAs were predicted separately by TargetScan, PITA as well as miRNA.org databases (Fig. 2C). 4,364 identical target genes were predicted in three different databases. The putative cellular process or associated functional pathways of these genes were further analyzed using the DAVID bioinformatics tool (KEGG). The top 12 canonical pathways were presented. The significant pathways were enriched in PI3K-Akt signal pathway, mTOR signal pathway, FoxO signal pathway and MAPK signal pathway (Fig. 2D). We also categorized these identical target genes based on the GO annotation. The results showed that cytoplasm was the main location of the miRNA-target genes and the top significant function was associated with protein binding. In addition, DNA-templated transcription was the most important biological process (Additional file4:Fig. S1).

**qPCR verification of differentially expressed miRNAs in myocardial EVs and cardiomyocytes with normoxia and intermittent hypoxia treatment**

Based on the criteria with 2-fold cutoff and p value less than 0.05, 16 differentially expressed miRNAs with homologous sequence within mouse and human including 8 up-regulated miRNAs and 8 downregulated miRNAs (Table 1) were selected for further verification study. Except for the detection in EV, qPCR assay was also used to measure the expression level of 16 miRNAs in normoxia (Nor) and IH-treated cardiomyocytes (Fig. 3 A-F and Additional file5:Fig. S2). Consistent with the miRNA microarray chip assay, qPCR results showed 11 verified miRNAs, including miR-125b-5p, miR-1a-3p, miR-22-3p, miR-23a-3p, miR-29a-3p and miR-494-3p which were significantly increased in EVs from IH-treated cardiomyocytes, and miR-101a-5p, miR-16-23p, miR-181b-5p, miR-214-5p and miR-448-5p which were significantly decreased. Meanwhile, we found the expression of miR-181-5p, miR-1a-3p and miR-494-3p had the identical tendency in IH-exposed cardiomyocyte as their expression in EVs from IH-treated cardiomyocyte, whereas the expression of miR-16-2-3p has the opposite tendency (Fig. 3 A-F). Of note, miR-30c and miR-129b-5p were expressed in a large amount in cardiomyocytes, but little in EVs. Whereas, miR-504-3p was abundantly expressed in EVs rather than cardiomyocytes (Additional file6:Fig. S3).
Bioinformatics analysis of target myocardial EV miRNAs

Heat map showed the expression files of 11 verified miRNAs including five downregulated miRNAs and six upregulated miRNAs (Fig. 4A). Venn map showed that the 11 miRNAs had over 1600 common target genes predicted by three databases (TargetScan, microRNA.org and PITA) (Fig. 4B). KEGG pathway analysis revealed that PI3K-Akt signal pathway and FoxO signal pathway were the top two significantly different pathways. Six upregulated miRNAs (miR-22-3p, miR-494-3p, miR-23a-3p, miR-125b-5p, miR-29a-3p and miR-1a-3p) were involved in PI3K-Akt signal pathway, and among them, 4 miRNAs (miR-125b, -23a, -22, -1a) were predicted directly regulating Akt expression by 3 target gene database analysis (Fig. 4C-D). Go enrichment revealed the possible function of myocardial EVs under IH condition. The cellular component analysis revealed that the miRNA target gene was principally located in nucleus. The molecular function analysis revealed that the miRNA target genes were involved primarily in protein binding. The top significant biological process in which the target gene participated was transcriptional regulation (Additional file7: Fig. S4). The predicted targets of the selected six miRNAs (miR-22-3p, miR-494-3p, miR-23a-3p, miR-125b-5p, miR-29a-3p and miR-1a-3p) were separately predicted by TargetScan database and then shown by visual graphs (Additional file8: Fig. S5).

The effect of EV from IH-exposed cardiomyocytes on endothelial-dependent relaxation

Considering the enriched function of differentially expressed EV miRNA was mainly concentrated in Akt pathway, we hoped to explore the effect of IH EV (EV from IH-exposed cardiomyocytes) on endothelial function since Akt/eNOS signaling axis is a well-established way leading to endothelial-dependent relaxation[14]. Consistent with database prediction, total Akt (normalized to GAPDH), phosphorylated Akt (normalized to t-Akt) and phosphorylated eNOS (normalized to t-eNOS) were significantly downregulated in IH myocardial EV-treated HUVES compared to its counterpart (Fig. 5A-B). Correspondingly, functional study showed that acetylcholine-caused dose-dependent relaxations in precontracted aortic rings were dramatically impaired in aortas with IH myocardial EV-48 h treatment (Fig. 5C). Whereas, IH myocardial EVs did not affect endothelial independent relaxation induced by NO donor, SNP (sodium nitroprusside) (Fig. 5D).

Discussion

In the present study, we carried out miRNA comparison assay in EVs from IH- or normoxia-treated cardiomyocytes and obtained two novel findings. First, we identified 63 miRNAs in EVs from IH-treated myocardial myocytes with different expression compared to control group. Among them, 11 homologous miRNAs were verified with the identical expression alteration as they appeared in the miRNA chip assay. Moreover, we revealed that the expression of miR-181-5p, miR-1a-3p and miR-494-3p has the identical tendency in IH-exposed cardiomyocyte as their expression in EVs from IH-treated cardiomyocyte, whereas the expression of miR-16-2-3p has the opposite tendency. Second, we found IH EVs derived from IH-treated cardiac myocytes effectively inhibited Akt/eNOS signal and significantly impaired endothelial-dependent relaxation (Fig. 5E).
Extracellular vesicle, as a nanovesicle secreted by various types of cells, encapsulates and transfers a wide range of functional proteins, lipids and nucleic acids to recipient cells to mediate intercellular communication[15]. Growing evidences indicated that EV participates in OSAS- or IH-associated cardiovascular diseases through transferring the altered EV components[16, 17]. Landscape studies of EV-miRNA changes under OSAS or IH are bound to provide possible insights into the potential molecular mechanisms of IH-related cardiovascular diseases. For instance, intermittent hypoxia altered circulating exosome miRNA cargos that promote the increased permeability and dysfunction of endothelial cells[18]. However, a systematic comparison of the miRNA difference in EVs secreted by cardiomyocytes under intermittent hypoxia and normoxia has not yet been reported. In this study, we identified 63 miRNAs with different expression in EVs from IH-treated cardiomyocytes. Among them, 11 homologous miRNAs were verified to have the same expression alterations as them appeared in miRNA chip assay. Moreover, 6 miRNAs with up-regulated expression in IH myocardial EVs (miR-125b-5p, miR-1a-3p, miR-22-3p, miR-23a-3p, miR-29a-3p and miR-494-3p) were predicated mainly involved in Akt pathway according to KEGG bioinformatic functional analysis. And further prediction by Targetscan database suggested Akt1/Akt2/Akt3 were the direct target genes of miR-125b, -23a, -22, -1a. Akt/eNOS signaling axis is a well-established way leading to endothelial-dependent relaxation[14]. As expected, we proved the adverse effect of EV derived from IH-treated cardiomyocyte on endothelial function as well as the reduced expression of t-Akt, p-Akt and p-eNOS in HUVEC with IH myocardial EV treatment. In addition to the endothelial function, PI3K/Akt signaling pathway was also reported mediating cardioprotective effect in cardiomyopathy induced by many diseases[19]. For example, the protective effects of tanshinone IIA on diabetic cardiomyopathy[20], Sulforaphane on hypertrophic cardiomyopathy[21], as well as Darbepoetin alfa on autoimmune cardiomyopathy were all mediated by enhanced Akt activity[22]. Especially, enhanced PI3K/Akt activation reversed the adverse effects of hypoxia-ischemia damage in cardiomyocytes by promoting certain survival-related proteins and anti-apoptotic proteins[23]. PI3K/Akt pathway was also involved in the beneficial effect of resveratrol on chronic intermittent hypoxia-induced cardiac hypertrophy[24]. Moreover, molecular derived from stem cells EVs also participated in cardiomyocyte survival through activating PI3k/Akt pathway[25]. All the previous reports together with our findings suggests that differentially expressed miRNAs in EVs from IH-treated cardiomyocytes may be involved in IH-triggered cardiovasculopathy via directly regulating Akt activity.

In addition to the predicted functions relevant to PI3K/Akt pathway, the differentially expressed EV miRNAs identified in our results have several known pathological functions in cardiovascular system. For example, previous findings revealed a pivotal role for miR-125b-5p in regulating cardiomyocyte survival during acute myocardial infarction[26] and EVs-derived miR125b-5p under hypoxia-conditioned facilitated ischemic cardiac repair by ameliorating cardiomyocyte apoptosis[27]. Moreover, recent researches indicated miR-22-3p was a key molecule in regulating vascular smooth muscle cell proliferation and migration by targeting HMGB1[28] and a promising therapeutic target for atherosclerosis treatment[29]. EV miR-23a-3p from mesenchymal stem cells attenuated myocardial injury through suppressing DMT1 expression[30]. And miR-29a-3p played a protective role in TNFa-induced endothelial dysfunction[31]. Therefore, the myocardial EV miRNA components and their variations under IH status will help us to
deeply understand the possible mechanism of IH-induced cardiovasculopathy, or help to reveal the function of myocardial EV in IH or OSAS-related cardiovascular diseases or even other relevant complications[32].

Except for the validation of EV miRs with different expression, we also measured the expression of corresponding miRs in IH-treated cardiomyocyte. We found that the expression amount of miR-129b-5p and miR-30c-5p was dramatically higher in cardiomyocytes than that in myocardium EVs, indicating that these miRs mainly exert their function in cardiomyocytes rather than in EVs. On the contrary, the amount of miR-504-3p was significantly higher in EVs than that in cardiomyocytes, suggesting miR-504-3p possibly functions mainly by EV mediation. In general, EVs contain multifarious biomolecules with the similar express profile with their parent cell contents and provide crucial information about an individual’s physiological context[33]. Consistent with this view, in our results, the expression of miR-181-5p, miR-1a-3p and miR-494-3p had the identical tendency in IH-exposed cardiomyocyte as their expression in EVs from IH-treated cardiomyocyte. Otherwise, it also has been well recognized that the sorting of EV cargos is a precisely regulated process and some molecules with opposite expression propensity in EVs and their donor cells under specific status[34]. Our results supported the opinion as well by demonstrating that miR-16-2-3p expression was increased by IH treatment in cardiomyocytes while was inhibited in EVs from IH-treated cardiomyocytes, indicating that most of miR-16-2-3p was kept in cardiomyocyte to play its role under IH status rather than secreted from cardiomyocytes to EVs.

This study found many differentially expressed miRNA in IH myocardial EVs and predicated their function relevant to Akt pathway, whereas, we only restricted our studies to examine the contribution of IH myocardial EVs on HUVECs and endothelial function. Other possible cardiovascular effect of IH myocardial EVs cannot be ignored in explanation of IH-or OSAS-triggered cardiovascular diseases since Akt is a pivotal regulator in a wide range of cellular functions. Moreover, we do have proved that IH myocardial EVs can impair endothelial function and provided the possible miRNAs involved during the process, while we did not precisely illustrate the specific miRNA of EVs in this regulation mechanism. Therefore, future validation investigation is merited to solve these questions.

Conclusions

In conclusion, this study for the first time offers a landscape view of myocardial EV-miRNA changes induced by intermittent hypoxia and reveals their possible function on endothelium, which not only elucidates the possible mechanism of IH-or OSAS-promoted cardiovascular diseases, but also provides useful diagnostic and therapeutic clues in the ongoing battle against IH- or OSAS-related cardiovascular complications.

Abbreviations

IH: Intermittent hypoxia ; OSAS: obstructive sleep apnea syndrome ; Nor: Normoxia ; EV: Extracellular vesicle ; HUVES: human umbilical vein endothelial cells ; α-SA: α-sarcomeric actin ; SNP: sodium
nitroprusside;

**Declarations**

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**Author contributions**

Yu Li and Huina Zhang carried out most of the experiments and wrote the manuscript. Yunhui Du and Lu Peng helped the cell culture experiments. Yanwen Qin and Huirong Liu provided suggestions for this research. Xinliang Ma guided the experimental design and edited the manuscript. Yongxiang Wei provided the research equipment for all the experiments and financed the study. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated in this study are included in the manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Declaration of Competing Interest**

The authors declare no conflict of interest.

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**Tables**

**Table 1.** IH myocardial EV miRNAs with significant different expression and homologous sequence in human and mouse.

| miRNA downregulation | miRNA upregulation |
|----------------------|--------------------|
| Name | Fold change (log2) | p.value | Name | Fold change (log2) | p.value |
| let-7j | -1.5043 | 0.007463 | miR-125b-5p | 2.450103 | 0.002446 |
| miR-101a-5p | -4.75453 | 0.033366 | miR-133b-3p | 1.137319 | 0.0003338 |
| miR-129b-5p | -1.52039 | 0.041677 | miR-1a-3p | 1.500538 | 0.034398 |
| miR-16-2-3p | -1.45458 | 0.02208 | miR-22-3p | 1.212431 | 0.021577 |
| miR-181b-5p | -2.94874 | 0.000135 | miR-23a-3p | 2.188835 | 0.002125 |
| miR-214-5p | -1.89447 | 0.039164 | miR-29a-3p | 1.887891 | 0.005642 |
| miR-448-5p | -3.77987 | 0.033781 | miR-30c-5p | 2.450475 | 0.010812 |
| MiR-504-3p | -1.17541 | 0.012039 | miR-494-3p | 1.099279 | 0.005468 |

**Figures**
Characterization of myocardial EVs. (A) EVs isolated from mouse primary cardiomyocytes were imaged by transmission electron microscope after negative staining, bar, 200 nm. (B) Cardiomyocytes and cell culture medium fractions including EVs were processed for Western blotting assay with cardiomyocyte specificity marker α-SA, exosomal marker antibodies against CD63, CD81; cytosol protein antibodies against TSG101 and Alix; as well as endoplasmic reticulum marker antibody against calnexin. Silver
staining showed the protein loading amount and protein profile of each sample. (C) Size distribution of EVs was detected by NS300 system (Malvern, UK). Blue line, the size distribution of EV derived from normoxia (Nor)-treated cardiomyocytes. Red line, the size distribution of EV derived from intermittent hypoxia (IH)-treated cardiomyocytes. (D) The concentrations of EVs from cardiomyocytes after IH and Nor treatment for 24 hours were evaluated by NS300 system (n=3). p value, *** < 0.001.

Figure 2

MicroRNA chip assay of myocardial EVs from Nor- or IH-treated cardiomyocytes. (A) Differentially expressed miRNAs were selected according to microRNA chip assay of EVs from IH- and Nor-treated cardiomyocytes. (B) Volcanic map showed the 63 EV miRNAs with different expression. (C) Venn
diagram showed the number of overlapped target genes of differentially expressed EV miRNAs predicted separately by TargetScan, PITA as well as miRNA.org databases. (D) KEGG pathway predicted the top enriched functions of the identical target genes of 63 EV miRNAs with different expression.

Figure 3

Verification of differentially expressed miRNAs in myocardial EVs and cardiomyocytes with normoxia and intermittent hypoxia treatment. The relative expression of miR-101a-5p and miR-181b-5p (A), miR-16-2-3p
and miR-1a-3p (B), miR-214-5p and miR-448-5p (C), miR-29a-3p and miR-125b-5p (D), miR-22-3p and miR-494-3p (E), miR-23a-3p (F) in myocardial EVs and cardiomyocytes under IH and Nor treatment was shown graphically (n=3). Asterisks indicate statistical significance. p value, *< 0.05; **< 0.01; *** < 0.001; **** < 0.0001.
Bioinformatic analysis of validated miRNAs and functional study of IH myocardial EVs on endothelial function. (A) Heat map analysis of with different expression. (B) Venn analysis showed the overlapped predicted target genes of 11 validated myocardial EV miRNAs. (C) KEGG pathway analysis of 11 validated myocardial EV miRNAs. (D) The microRNA target gene network analysis of 6 upregulated IH myocardial miRNAs demonstrated the common target of Akt.

Figure 5

EV from IH-exposed Cardiomyocytes Impairs Endothelial-dependent Relaxation. (A) Western blotting detected protein levels in HUVEC cells with Nor myocardial EVs and IH myocardial EVs treatment (n=3). (B) Quantitative analysis of t-Akt(normalized to GAPDH), p-Akt t(normalized to Akt), t-eNOS (normalized to GAPDH) and p-eNOS (normalized to eNOS). (C) Dose-response of endothelial-dependent relaxation was measured for cumulative increments of 10-9-10-5 M acetylcholine in 24h and 48h (n=4). (D) Endothelial-independent relaxation was measured in response to the addition of 10-9-10-5 M SNP (sodium nitroprusside). (E) Schematic illustration of the proposed role of IH myocardial EV in endothelial dysfunction. EVs from IH-exposed cardiomyocytes dramatically impaired endothelium-dependent relaxation and inhibited Akt/eNOS pathway in endothelial cells through delivering Akt regulating miRs. The data are shown as mean±SD (n=4). p value, *** < 0.001.
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