Effect on Intermittent Hypoxia on Plasma Exosomal Micro RNA Signature and Endothelial Function in Healthy Adults

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Study Objective: Intermittent hypoxia (IH) is associated with increased risk of cardiovascular disease. Exosomes are secreted by most cell types and released in biological fluids, including plasma, and play a role in modifying the functional phenotype of target cells. Using an experimental human model of IH, we investigated potential exosome-derived biomarkers of IH-induced vascular dysfunction.

Methods: Ten male volunteers were exposed to room air (D0), IH (6 h/day) for 4 days (D4) and allowed to recover for 4 days (D8). Circulating plasma exosomes were isolated and incubated with human endothelial monolayer cultures for impedance measurements and RNA extracted and processed with messenger RNA (mRNA) arrays to identify gene targets. In addition, immunofluorescent assessments of endothelial nitric oxide synthase (eNOS) mRNA expression, ICAM-1 cellular distribution were conducted.

Results: Plasma exosomal micro RNAs (miRNAs) were profiled. D4 exosomes, primarily from endothelial sources, disrupted impedance levels compared to D0 and D8. ICAM-1 expression was markedly upregulated in endothelial cells exposed to D4 exosomes along with significant reductions in eNOS expression. Microarray approaches identified a restricted and further validated signature of exosomal miRNAs in D4 exosomes, and mRNA arrays revealed putative endothelial gene target pathways.

Conclusions: In humans, intermittent hypoxia alters exosome cargo in the circulation which promotes increased permeability and dysfunction of endothelial cells in vitro. A select number of circulating exosomal miRNAs may play important roles in the cardiovascular dysfunction associated with OSA by targeting specific effector pathways.

Keywords: cardiovascular disease, circulating miRNAs, endothelium, exosomes, experimental human model, healthy human volunteers, intermittent hypoxia, sleep apnea


Significance

Intermittent hypoxia in healthy humans mimicking sleep apnea for 4 days reversibly alters the cargo and functional properties of circulating exosomes and their effects on endothelial function and gene targets. Therefore, improved understanding of the pathways activated by intermittent hypoxia may enable delineation of biomarkers and therapeutic targets for sleep apnea-associated morbidities.

INTRODUCTION

Obstructive sleep apnea (OSA) is a highly prevalent condition affecting a large portion of the adult population.1–4 The adverse health implications of OSA have emerged over the past few decades, whereby an increased risk for both cardiovascular and metabolic morbidities have been consistently identified in large epidemiological cohorts among OSA patients.5–7 Furthermore, evidence of endothelial dysfunction, an early event preceding the clinical manifestations of cardiovascular disease, is apparent in patients with OSA.8–10 In animal models of OSA, intermittent hypoxic exposures as well as long-term sleep fragmentation induce endothelial dysfunction,11,12 thereby indicating a potential causal relationship between OSA and the disrupted structural and functional integrity of the vasculature. In previous studies, we have exposed young healthy adults to intermittent hypoxia (IH), a potential major factor contributing to the pathogenesis of OSA-related comorbidities, and clearly demonstrated the presence of perturbations in endothelial function that are reversible upon termination of the IH exposures.13–16 However, the potential mechanisms leading to endothelial dysfunction remain unclear.

Extracellular vesicles, particularly exosomes, are undoubtedly one of the most groundbreaking discoveries in cell biology in recent decades. These endosome-derived vesicles (30–100 nm) are actively shed by cells in the context of extremely regulated processes of cellular housekeeping and intercellular communication,17 and can be readily isolated from human plasma.18 Recent evidence indicates that exosomes derived from specific endothelial progenitor cell lineages can protect the endothelium against acute hypoxia–reoxygenation injury,19 a finding that may account for the heterogeneity of vascular dysfunction in children with OSA.20 Exosomes can carry signaling molecules to activate local target cells in tissues, including cardiomyocytes, endothelial cells, fibroblasts, and stem cells, as well as distant organs, e.g., bone marrow.21 Exosomes have recently emerged as novel elements of intercellular communication in the cardiovascular system. In particular, exosomal micro RNAs (miRNAs) play an important role in heart disease development and progression through the modulation of intercellular communication in different cardiac cell types.22 Furthermore, exosomes shed by endothelial cells have been implicated in processes involved in atherosclerosis and cardiovascular disease.23–25 We hypothesized that
plasma-derived exosomes from human subjects exposed to IH may recapitulate *in vitro* the previously identified *in vivo* endothelial dysfunction, and further posited that the cargo of such exosomes would provide a unique miRNA signature that may underlie putative relevant endothelial cell targets.

**METHODS**

**Subjects and Protocols**

Ten healthy male subjects participated in the study. All subjects were nonsmokers, were not taking any medications, and had no history of cardiovascular, cerebrovascular, or respiratory disease. All participants were residents of Calgary, Alberta, Canada. Their mean (± standard deviation) age was 29.3 ± 1.7 y, and their body mass index was 25.6 ± 0.4 kg/m². The research study was approved by the Conjoint Health Research Ethics Board at the University of Calgary, and written informed consent was obtained from each subject prior to participating in the study. In addition, the study was also approved by the Human Subject Committee at the University of Chicago (protocol #: 10-702-A-CR004). The experimental human model of IH, along with the details of the protocol, have been previously described. Briefly, subjects were exposed in a chamber where the gas composition was altered by either adding nitrogen or oxygen, and by adding or removing carbon dioxide. On days of exposure to IH, the gas composition within the chamber was set at a level resulting in a PETO2 of 45.0 mmHg. Periods of normoxia were elicited by delivering 100% oxygen to the subject through an oxygen diffuser (Oxymask, Southmedic, Barrie, Ontario, Canada) worn on the mouth. The oxygen flow rate was set to produce a PETO2 of 88.0 mmHg. When oxygen flow through the diffuser was zero the subject breathed the gas composition of the chamber, which corresponded to a PETO2 of 45.0 mmHg. During the recurring hypoxic episodes, respired gas was sampled from a nasal cannula and analyzed by a dual oxygen and carbon dioxide analyzer (NormocapOxy, Datex-Ohmeda, Louisville, CO, USA) for PO2 and PCO2. The identical experimental setup was used during the baseline measurements and sham protocol, except that the chamber was not hypoxic and room air was delivered through the oxygen diffuser rather than 100% oxygen. Details on subjects and current study experimental design are illustrated in Figure 1.

**Exosome Isolation, Labeling, and Characterization**

Exosomes are characterized by their preserved size, density, and by the presence of specific protein markers. Exosomes were isolated from plasma using the Total Exosome Isolation Kit according to the manufacturer’s protocol (Life Technologies, Carlsbad, CA, USA), and further characterized as previously reported. Briefly, plasma was centrifuged at 2000×g for 20 min to remove cell debris. The supernatants were collected and 0.2 volume of the Total Exosome Isolation Reagent was added. The mixtures were incubated at 4°C for 30 min followed by centrifugation at 10,000×g for 10 min, and pellets were solubilized in 1× phosphate buffered saline (PBS).
Endothelial Cell Culture

Human microvascular endothelial cells (HMVEC) were purchased from Lonza (catalog #: CC-2543; Allendale, NJ, USA). Cells were maintained in endothelial growth medium (EGM-2-MV; Clonetics) supplemented with 5% fetal bovine serum (FBS; Clonetics, Lonza, Walkersville, MD) and incubated at 37°C and 5% CO2 in cell culture incubator. For continuous passage, the cells were trypsinized and centrifuged at 220g for 7 min, diluted, and replated at appropriate densities. All cells were used before passage 4–6.

bEnd3 cells (American Type Culture Collection, Manassas, VA, USA), an immortalized murine endothelial cell line derived from primary murine brain microvasculature transformed by polyomavirus middle T antigen, were purchased. Cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 4 mmol/L glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin, and pH 7.4, and cells were incubated at 37°C and 5% CO2. For continuous passage, the cells were trypsinized and centrifuged at 150g for 7 min, and replated at appropriate densities.

Exosome Cell Sources

Isolated exosomes from adult subjects (D0, D4, and D8) were subjected to ImageStream imaging cytometer (Millipore/Amnis, Seattle, WA) for detection of their cell sources. The ImageStreamX Mark II Imaging Flow Cytometer combines the speed, sensitivity, and phenotyping abilities of conventional state-of-the-art flow cytometry with the detailed imagery and functional insights of digital microscopy. This unique combination enables a broad range of applications that would be impossible using either technique alone. This instrument produces multiple high resolution images of every cell directly in flow, including brightfield and darkfield (SSC), and enables up to 10 fluorescent markers with a sensitivity far exceeding conventional flow cytometers. Each gated population was interrogated via the image gallery to determine the upper and lower limits of exosomes size and shape. Briefly, 100 μL of exosomes were stained with appropriately titrated antibodies (BioLegend, San Diego, CA) as follows: CD14 Alexa 488, CD309 PE, CD133 PECy7, CD34 BV421, CD31 BV605, CD42b APC, CD45 APCy7. An antibody cocktail of all 7 antibodies were created and queued up to 100 μL per sample by adding 45 μL per sample of Brilliant Stain Buffer (BD Biosciences, San Jose, CA). A cocktail of diluted antibodies was passed through a 0.1-μm filter and 100 μL was added to the blocked sample for 60 min at 4°C in dark. After incubation, samples were washed in 5 mL of 0.1 μm filtered wash buffer (PBS, 0.1% fetal bovine serum, BSA), and stained exosomes were centrifuged at 17,000g for 10 min. Supernatants were removed and the pellet was resuspended in exactly 100 μL of 0.1 μm filtered wash buffer (final volume). The ImageStreamX image cytometer (Millipore/Amnis) uses the 60x objective and the high-sensitivity fluid flow rate setting. Excitation of the various fluorophores was performed by the four high-powered lasers on the system, specifically, 90 mW 405 nm, 100 mW 488nm, 200 mW 561 nm, and 120 mW 640 nm laser. Compensation was done using VersaComp beads (Beckman Coulter, Miami, FL, USA) stained with the same antibodies used in the assay. In addition, exosome size verification and matching was accomplished using 500 size-appropriate beads of each color, and an autocompensation was done using the IDEAS 6.0 data analysis software (Millipore/Amnis).

Electric Cell-Substrate Impedance Sensing

Endothelial cells were grown in DMEM media containing 2% FBS for 24 h. Cells were seeded (30,000 cells) and grown to confluence into electric cell-substrate impedance sensing (ECIS) arrays as a single confluent monolayer. Exosomes were added in duplicate wells and placed into the ECIS instrument (http://www.biophysics.com/products-ecisz0.php) for continuous monitoring up to 24 h. The ECIS array enables assessment of morphological cell changes, cell locomotion, and other behaviors directed by the cell’s cytoskeleton. ECIS uses 250 μm-diameter gold film electrodes deposited on the bottom of cell culture dishes and measures the electrode impedance. As cultured cells attach and spread on the electrode surface, impedance is altered, and serves as a measure for disruption of the endothelial cellular junction. This method is based on measuring noninvasively the frequency-dependent electrical impedance of cell-covered gold-film electrodes along the time course of the experiment. The baseline was established using culture medium (300 μL/well-1) alone and compared with values obtained using electrodes covered with a monolayer of cells in 500-μL medium.

Immunofluorescent Staining

Confluent human endothelial cell monolayers were grown on cover slips for 24 h in DMEM media containing 5% FBS. Cells were washed with DMEM media containing 2% FBS. Isolated exosomes from 6 subjects were added individually to cover slips for 24 h for each experimental condition. Cells were fixed with 4% (w/v) paraformaldehyde in PBS for 20 min at room temperature and then washed again with PBS. The cell membranes were permeabilized by incubation with 0.25% (v/v) Triton-X-100 in PBS for 10 min at room temperature. After washing with PBS the samples were blocked with 3% (w/v) bovine serum albumin in PBS for 45 min at room temperature to block unspecific binding sites, and followed by overnight incubation at 4°C with polyclonal ICAM-1 antibody (1:250) Santa...
Cruz Biotechnology, Inc., Dallas, TX). Alexa 488 or Alexa-594 were used as secondary antibodies (1:400, 2 mg/mL; Life Technologies, Grand Island, NY, USA) and nuclear staining with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride; 1:1000; Life Technologies) were performed. Appropriate controls and pre-adsorption experiments were performed to ascertain the specificity of the staining. Images were captured with a Leica SP5 Tandem Scanner Spectral 2-photon confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL) with a 63× oil-immersion lens.

**Monocyte Adhesion Assays**

The experimental protocols were approved by the Institutional Animal Use and Care Committee and are in close agreement with the National Institutes of Health *Guide in the Care and Use of Animals*. Transgenic B6.Cg-Tg (ACTB-mRFP1)F1Hadj/J mice (termed RFP) weighing 22–25 g, were purchased from Jackson Laboratories (Bar Harbor, Maine), housed in a 12 h light/dark cycle (light on 07:00 am to 19:00) at a constant temperature (24 °C) and allowed access to water and food *ad libitum*. RFP mice express the red fluorescent protein (DsRED-T3) under the control of a chicken β-actin promoter and cytomegalovirus enhancer. All of the tissues from this transgenic line, with the exception of erythrocytes and hair, are red under blue excitation light. Transgenic RFP male mice were sacrificed and used to isolate monocytes. Bone marrow cells were flushed from femur and tibia using a syringe equipped with a 23-gauge needle. Cells suspensions were filtered through a 70-µm mesh nylon strainer, and centrifuged at 300×g for 6 min. The cells were counted using automated cell counter (Cellometer, Nexcelom Bioscience, Lawrence, MA, USA), and the concentration of BMC was prepared to be 1 × 10⁶ cells per mL. The samples were incubated with EasySep (Vancouver, BC) Biotin Selection Cocktail at 100 µL/mL of cells (e.g. for 2 mL of cells, add 200 µL of cocktail) in the refrigerator (2 to 8°C) for 15 min. Magnetic particles (50 µL/mL) were added and incubated in the refrigerator for 10 min to enable magnetically labeled unwanted cells to remain bound inside the original tube as held by the magnetic field of the EasySep Magnet. First, exosomes from the human subjects were added to a primary murine endothelial cell monolayer (bEnd3) and incubated for 24 h. Then, monocytes (4 × 10⁶) derived from RFP mice were added to the cell culture for only 30 min at 37°C, and then washed to reveal adherent monocytes. The cells were washed with cold PBS buffer three times to remove unbound RFP monocytes. Finally, the RFP cells remaining in the culture plates (adherent monocytes) were counted on fluorescent microscope.

**MiRNA Isolation**

Total RNA including miRNA was extracted from isolated exosomes using miRNeasy Mini Kit-column-based system following the manufacturer’s instructions (Qiagen, Turnberry Lane, Valencia, CA, USA). Briefly, the exosome pellets were solubilized in 700 µL of Qiazol Column, and dried for 5 min after the last washing step before elution. Total RNA was eluted by adding 14 µL of DNAse-RNase-free water to the membrane of the spin column and incubating for 1 min before centrifugation at 13,000×g for 1 min at room temperature. The RNA quality and integrity was determined using the Eukaryote Total RNA Nano 6000 LabChip assay (Agilent Technologies, Santa Clara, CA) on the Agilent 2100 Bioanalyzer. The quality of miRNA was determined using Agilent Small RNA Kit according to the manufacturer’s protocol. Both total RNA and miRNA samples were quantified on a Nanodrop 2000 (Ambion, Austin, TX, USA).

**MiRNA Microarrays**

Each sample was prepared according to Agilent’s miRNA recommended approach using the one-color technique, and profiled on the Agilent human miRNA microarray (Agilent Technologies). Each array consisting of 60-mer DNA probes synthesized in situ that represents 2,006 human miRNA. Total RNA including enriched miRNA was dephosphorylated with calf intestine alkaline phosphatase (GE Healthcare Europe GmbH), denatured with dimethyl sulfoxide, and labeled with pCP-Cy3 using T4 RNA ligase (GE Healthcare Europe GmbH). The labeled RNAs were hybridized to custom human miRNAs microrrays 8x60K (Agilent, Santa Clara, CA). After hybridization and washing, the arrays were scanned with an Agilent microarray scanner using high dynamic range settings as specified by the manufacturer. Microarray results were extracted using Agilent Feature Extraction software (v12.0) to quantify signal intensities.

The quality control for each miRNAs was evaluated based on their Agilent Feature Extraction quality control report. MiRNA microarray data were log-base 2 transformed and quantile normalized. The normalized data was expressed as the difference of log of g processed signal (Agilent Feature Extraction). Undetected probes were excluded from further analysis. Background-subtracted intensities were normalized for detected miRNA probes using the quantile method across all miRNA microarray experiments as described by Gharib et al. The false discovery rate was controlled at 5% to correct for multiple comparisons.

**miRNA Target Predictions and Network Construction**

Gene targets for differentially expressed miRNAs were initially computationally predicted using established miRNA target-prediction programs: MicroInspector, miRanda, PicTar, RNA22, RNAhybrid, and TargetScan. In order to improve the reliability of the miRNA targets, only target genes predicted by at least three of the programs were selected. The predicted genes of individual miRNA were uploaded to the online DAVID program (http://david.abcc.ncifcrf.gov/) for their functional annotation and clustering analysis. The predominant biological pathways for the selected miRNAs were identified.

Molecular targets for each miRNAs were retrieved and the validated miRNA-target interaction network was obtained from the CyTargetLinker plug-in in the Cytoscape environment. CyTargetLinker, a Cytoscape apparatus, provides an extensible framework to integrate different regulatory interactions from databases including MicroCosm, TargetScan, miTarBase, and ENCODE. The network containing interactions between differentially expressed (DE) DE-miRNA and...
putative targets was constructed and visualized using Cytoscape (http://cytoscape.org).

mRNA Microarrays for Assessment Exosome Targets

Total RNA was isolated from HMVEC cells, (total n = 18) corresponding to the following conditions (n = 6/condition): (1) cells treated with D0-derived exosomes, (2) cells treated with D4 exosomes, and (3) cells treated with D8-derived exosomes using miRNeasy Mini Kit-column-based system following the manufacturer’s instructions (Qiagen). Briefly, HMVEC cells were maintained in endothelial growth medium with 5% fetal bovine serum (FBS; Clonetics) and incubated at 37°C and 5% CO₂ in cell culture incubator until confluence. Cells were washed with DMEM media containing 2% FBS and exosomes were added for 24 h. Total RNA was purified and processed for labeling using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Santa Clara, CA) and hybridized with whole-genome human Agilent microarrays (8 × 60 K). Equal quantities of total RNA were labeled with each reaction containing 25 ng of total RNA and 2 µL (34 pg) of RNA spike-in control. The quality of each complementary RNA sample was evaluated using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Each sample was hybridized to an Agilent oligonucleotide microarray for all of the 18 independent experiments. The microarray slides were scanned using Agilent dual-laser Microarray Scanner and the digitized images were acquired and processed using Agilent Feature Extraction (FE) software v.12.0.

Background-subtracted intensities were normalized using the quantile method across all remaining 18 microarray experiments. The background corrected data were normalized between arrays using cyclic loess method. We applied limma moderated t-test to detect differentially expressed genes, considering the batch effect caused by different arrays as covariates in the linear model. P values were adjusted by Benjamini-Hochberg method. Differentially expressed genes (DEGs) were identified either using false discovery rate of 0.05 or log-fold change of 1.5 and FDR of 0.05.

qRT-PCR Validation

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed for selected mRNAs and miRNAs using ABI PRISM 7500 System (Applied Biosystems, Foster City, CA). For RNA, cDNA was synthesized using 250 ng of total RNA using a High-Capacity cDNA Archive Kit (Applied Biosystems). Human TATA-box binding protein (TBP gene) was used as a reference gene to normalize the expression ratios. The cycle number (Ct) values were averaged and the difference between the TBP Ct and the gene of interest Ct were calculated to calculate the relative expression of the gene of interest, and the data was normalized to D0.

miRNAs were reverse transcribed with looped miRNA-specific reverse transcription (RT) primers (Applied Biosystems) using the TaqMan miRNA mouse assays according to the manufacturer’s protocol. Briefly, RT reactions were performed in a volume of 15 µL and each reaction contained 10 ng of enriched miRNA. RT reactions were performed on a GeneAmpPCR System 7500 (Applied Biosystems) with the following conditions: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and 4°C on hold. All TaqMan assays were run in triplicate using TaqMan Universal PCR Master Mix II without UNG (Applied Biosystems). Real-time PCR (RT-PCR) cycling conditions consisted of 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. The qRT-PCR results were normalized against an internal control (RNU6), and then expressed as fold changes. All experiments were performed in triplicates. The Ct values were averaged and the difference between the RNU6 (Avg) and the gene of interest Ct (Avg) were calculated (Ct-diff) to calculate the relative expression of the gene of interest using the 2ΔΔCT method.

Data Analysis

All data were expressed as means ± standard deviation. We performed analysis of variance with Bonferroni multiple comparison tests to study differences among quantitative variables between D4 and D0. All analyses were conducted using SPSS software (version 21.0; SPPS Inc., Chicago, IL, USA). Comparisons between exosome-treated and untreated cells were performed using t-tests or one-way analysis of variance with Bonferroni multiple comparison tests. A value of P < 0.05 was considered statistically significant for all analyses.

RESULTS

Exosome Cell Sources

Exosomes originating from endothelial cells constituted the largest proportion of the plasma-derived exosomes among IH-exposed subjects (D4 group), and their concentrations were significantly higher in D4 compared to baseline D0, with no significant differences emerging after normoxic recovery (D8 group) and D0 (Figure 2). Similarly, higher exosome counts from endothelial cells, progenitor cells, monocytes, lymphocytes, and platelets were present in IH-exposed exosomes (D4 group) compared to baseline conditions (Figure 2).

Effect of IH-Derived Exosomes on Endothelial Barrier

Human endothelial cells were cultured in a single monolayer and exposed to exosomes from D0, D4, and D8. Normalized ECIS resistance values were continuously monitored, and revealed substantial decrements in monolayer resistance that were significantly more prominent for exosomes at D4, when compared to either D0 or D8 (Figure 3A and 3B; P < 0.002). Furthermore, D4-derived exosomes induced increased expression of ICAM-1 protein expression in endothelial cells, an effect that was not apparent at D8 (Figure 3C).

In addition, we quantified the mRNA expression of the eNOS (NOS3) gene in endothelial cells treated with plasma-derived exosomes from D0, D4, D8 conditions using qRT-PCR. As shown in Figure 4, eNOS expression was significantly decreased by D4-derived exosomes (D4 vs. D0: P < 0.003), and such effect was absent following normoxic recovery (D8 vs. D4: P < 0.01).

IH-Derived Exosomes and Monocyte-Endothelial Cell Adhesion

Next, we examined whether exosomes isolated from D0, D4, and D8 conditions and applied to naïve murine endothelial
Figure 2—Exosomal-cell sources in subjects at baseline (D0), after exposure to IH for 4 days (D4), and following 4 days of normoxic recovery (D8).

(A) Representative image from the ImageStream X imaging cytometer on one of the six subjects aimed at identifying and quantifying the relative proportion of exosomes derived from endothelial cells, endothelial progenitor cells, monocytes, and platelets cells. Please note that the panel on monocytes in the figure contains both leukocytes (left upper quadrant) and monocytes (right upper quadrant). Percentages are not shown because they are less than 0.1% of all counts. (B) Summary of six separate cytometric analyses of exosomes showing the relative proportion of exosomes derived from endothelial cells (D4 vs. D0: P < 0.01; D8 vs. D0: P < 0.05; D4 vs. D8: P < 0.05), endothelial progenitor cells (D4 vs. D0: P < 0.001; D8 vs. D0: P < 0.05; D4 vs. D8: P < 0.05), lymphocytes (D4 vs. D0: P < 0.001; D8 vs. D0: P < 0.05; D4 vs. D8: P < 0.05), monocytes (D4 vs. D0: P < 0.001; D8 vs. D0: P < 0.05; D4 vs. D8: P < 0.05), and platelets (D4 vs. D0: P < 0.01; D8 vs. D0: P < 0.05; D4 vs. D8: P < 0.05) (n = 6 subjects/group). Top right panel shows illustrative example of electron microscope image of isolated exosomes depicting the appropriate size of the vesicles.
cells in vitro would lead to adhesion of naïve RFP mouse-derived monocytes. Compared to exosomes isolated from D0 or D8, D4-derived exosomes markedly increased the adhesion of monocytes to murine endothelial cells (Figure 5; P < 0.0001 for D4 versus D0).

**Exosomal-miRNA Profiling and In Silico Prediction of Potential Targets**

miRNA array analyses of D0, D4, and D8 exosomal cargo (n = 6/group) were performed to identify uniquely differentially expressed miRNAs in D4. Principal component analysis plots revealed consistent group separation and categorical assignments, which were completely concordant with the actual three experimental groups, and further showed substantial differences in miRNA expression profiles (Figure 6A). Among the latter, six differentially expressed miRNAs were identified, (Table 1), and were validated using qRT-PCR.

As a next step, in silico target prediction tools were applied to identify potential targets for the six differentially expressed miRNAs, and revealed 2,028 putative genes. The gene ontology for those putative gene target predictions resulted into 51 genes ontology categories. Of those, there were 39 categories that were shared by all six miRNAs (Figure 6B). The top

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**Figure 3**—Effect of plasma-derived exosomes on Electric Cell-substrate Impedance Sensing (ECIS) and ICAM1 expression in human microvascular endothelial cells. Plasma-derived exosomes from subjects exposed to IH for 4 days (D4) induce cellular barrier disruption as identified via impedance sensing technology (ECIS) in an endothelial cell monolayer. Time-course of normalized endothelial resistance and their values were continuously monitored for up to 48 h after adding exosomes to the confluent endothelial monolayer. (A) Average of normalized resistance for empty exosomes (control-CTL), D0, D4, and D8. (B) Histograms for the groups at 20 h. The asterisk indicates statistical significance. D4 versus D0, P < 0.002, n = 8 subjects/group. (C) ICAM-1 immunohistochemistry of endothelial cells after treatment with exosomes. Exosomes from adult subjects exposed to IH for 4 days (D4) and normoxic recovery for 4 days (D8) compared to baseline (D0) were applied to human microvascular endothelial cells for 24 h. ICAM1 (green) and nuclei (DAPI blue) were immunostained with corresponding antibodies. Increased in ICAM-1 expression is apparent in IH, 4 days and decreased following normoxic recovery day 8. Images are representative of six subjects for each experimental condition. The scale bars for all the representative images are 25 μm.

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**Figure 4**—ENOS gene expression in endothelial cells. QRT-PCR analysis for eNOS (NOS3) gene expression in human endothelial cells treated with exosomes obtained from subjects prior to being exposed to intermittent hypoxia (IH) (D0), after 4 days of IH (D4), and after 4 days of normoxic recovery (D8). Data are presented as relative messenger RNA (mRNA) expression levels normalized to TBP gene or 18S. Bars and asterisk indicate significant differences for D4 versus D0 (P < 0.003), and D8 versus D0; (P < 0.01, n = 6 subjects/group).
five Gene Ontology (GO) categories included organelle, ion binding, cellular nitrogen compound metabolic process, biosynthetic process, and cellular protein modification process. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses for the putative 2,028 gene targets revealed the presence of 4 pathways, namely prion diseases (hsa05020), phosphatidylinositol signaling system (hsa04070), glycosphingolipid biosynthesis (hsa00603), and glioma (hsa05214) as shown in Figure 6C.

To gain a better understanding of the molecular mechanisms potentially involved in IH exposures, we explored the regulatory information networks (RIN) associated with IH exposures (D4 vs. D0) using publicly available data warehouses to predict the potential targets using CyTargetLinker plugin in Cytoscape (Table 1). In particular, validated target genes were automatically obtained from the miRTarBase, MicroCosm, and TargetScan repositories and visualized using Cytoscape. Each regulatory interaction in the subnetworks consists of two nodes, a regulatory component (miRNAs) and a transcription factor (target gene) connected by one edge. However, five of the six differentially expressed miRNAs in D4 samples showed no known RIN. The RIN for hsa-miR-383b-3p miRNA and mRNA is shown in Figure 7, and identified 23 regulatory information network validated targets as follows: (ARRB2, PATZ1, HNRRUL2, ODF2, GANAB, PDP2-HUMAN, PGAM1, RNF5, ZBTB26, RNF24, ZBTB7B, CAMK2G, TEAD2, EPHA10, POU2F2, PODN, RNF4, TIMELESS, SAMAD4, CREBL2, KPNB1, ECHDC3, and TNPO2).

Figure 5—Exosomes isolated after 4 days of IH attract more red fluorescent protein (RFP) murine monocytes in vitro. Exosomes from D0, D4 and D8 from adult subjects were added to a primary murine endothelial cell monolayer and incubated for 24 h. Monocytes (4 × 10⁵) derived from RFP mice were then added for 30 min, and then washed to reveal adherent monocytes. (B) Summary of findings for each of the experiments in which at least eight different fields per subject were counted and averaged for each subject (n = 6 subjects/group).
Using transcriptomic approaches, we identified a total of 333 DEGs in human endothelial cells exposed to exosomes from D4 conditions compared to D0, using 1.5-fold changes and Q-value < 0.05 criteria. Of the 333 genes, there were 131 whose expression was downregulated and 212 genes exhibiting increased expression. GO and KEGG pathway enrichment analyses were applied to the DEGs using DAVID tools and IPA software. Of note, the differences between D0 and D4 virtually disappeared at D8 (data not shown). Hierarchical clustering of

Table 1—List of differentially expressed micro RNAs in adult exposed to intermittent hypoxia for 4 days compared to baseline and their gene interactions as well as host genes.

<table>
<thead>
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<th>Host Genes</th>
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<td>NA</td>
<td>AEBP1 AE binding protein 1</td>
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Exosomal-miRNA Gene Targets in Endothelial Cells

Figure 6—Principal component analysis (PCA) plot of exosome micro RNA (miRNA) cargo from D0, D4, and D8. (A) PCA clustering of miRNA expression by subject group is denoted by different colors, and reveal complete and correct separation of miRNA expression across the adult subjects. (B) Gene Ontology (GO) for the six differentially expressed miRNAs in D4 versus D0. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for the same 6 miRNAs in D4 vs. D0, n = 6 subjects/group.
expression values for the 3 groups revealed accurate group assignment, indicating the presence of uniquely and significantly different expression patterns for the DEGs (Figure 8A). The top canonical pathways of the DEG are shown in Figure 8B and illustrate that the majority of the endothelial target pathways are related to the immune system. The top 10 networks associated with these DEGs (Table 2) highlight cell-to-cell signaling and interactions, and inflammatory response networks (Figure 8C).

To understand the functional categories of these endothelial DEGs, we first analyzed them using DAVID GO enrichment analysis. The top biological process with value of q < 0.05 were GO:0032502—developmental process, GO:0050896—response to stimulus, GO:0002376—immune system process, GO:0016043—cellular component organization, and GO:0022610—biological adhesion. The molecular functional terms that were significantly over-represented amongst the DEGs included: GO:0008009—chemokine activity, GO:0042379—chemokine receptor binding, GO:0004672—protein kinase activity, GO:0050780—dopamine receptor binding, and GO:0003714—transcription corepressor activity. Among the highly significant KEGG pathways for the differentially expressed miRNAs of interest: hsa04620:Toll-like receptor signaling pathway, hsa04010:MAPK signaling pathway, hsa04062:Chemokine signaling pathway, hsa04621:NOD-like receptor signaling pathway, hsa04512:ECM-receptor interaction, hsa04310:Wnt signaling pathway, hsa04660:T cell receptor signaling pathway, and hsa04350:TGF-beta signaling pathway.

**DISCUSSION**

In this study, we show that plasma-derived exosomes in otherwise healthy subjects exposed to 4 days of IH are constitutionally altered in their miRNA cargo, and exhibit the ability to induce endothelial dysfunction in vitro. We further demonstrate that such properties are reversed upon normoxic recovery of similar duration. Indeed, IH-exposed plasma exosomes disrupted the permeability of an endothelial monolayer (as indicated by decreases in ECIS signal), reduced the expression of eNOS, and induced increased ICAM-1 expression and adhesion to naïve monocytes. Exploratory analysis of the plasma exosomal miRNA cargo using microarrays revealed the presence of six differentially expressed miRNAs whose expression was selectively altered by the 4-day IH exposures, and such changes were nearly completely reversed following 4 days of normoxic (i.e., air-breathing) recovery. Furthermore, we explored both in silico putative targets for these differentially expressed exosomal miRNAs, and further identified their actual endothelial cell targets. Taken together, our findings suggest a vast repertoire of changes in exosome cargo that elicit altered endothelial function and gene expression, and may account for some of the cardiovascular morbidities associated with OSA.

The presence of altered concentrations, cell sources, and intrinsic properties of circulating microvesicles have been previously noted by several investigators in the context of OSA, whereby the ability of these microvesicles to induce coagulation, angiogenesis, and cell adhesion was noted, and globally believed to reflect a proatherogenic state. The enhanced pro-atherogenic state of OSA is further exemplified by not only the high cardiovascular morbidity burden of this condition, but also by the elevated prevalence of peripheral vascular disease in OSA patients, and multiple investigators have explored the vascular consequences of OSA in different settings.

Our study relied on healthy young adults subjected to a relatively brief IH exposure, such that we can only infer that more chronic exposures to IH in the context of OSA may further exacerbate the pathological changes induced by the disease, and hypothesize that the exosome-related activity on the endothelium will contribute to such processes, as illustrated by the current in vitro experiments. In addition, only one level of hypoxia severity was assessed, and we did not include women or subjects with known diseases such as obesity, such that potential differences on the effects of IH of varying severity in these populations will clearly need to be explored in future studies. We should caution, however, that the reversibility of the exosome-induced functional perturbations on the endothelium following a normoxic recovery period of the same duration as the initial IH-exposure does not necessarily imply that more chronic exposures will be accompanied by similar favorable outcomes. This issue will clearly need to be examined in
Figure 8—Heatmap cluster for the effects of exosomes obtained from D0, D4, and D8 in human endothelial cells. (A) Heatmap of differentially expressed genes as determined by experiments comparing the effects of exosomes in endothelial cells. The color key above the heatmap represents the different expression levels. (B) List of the most highly significant canonical pathways identified in exosomes from D4 versus D0 in human endothelial cells. The list of the top 10 differentially expressed networks in D4 versus D0 is shown in Table 2. (C) Representative of the network for cell-cell-signal and interactions, and inflammatory response, n = 6 subjects/group.

Table 2—List of top 10 differentially expressed endothelial cell gene networks after treatment with D4 versus D0-derived exosomes.

<table>
<thead>
<tr>
<th>ID</th>
<th>Networks</th>
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<tbody>
<tr>
<td>1</td>
<td>Cell-To-Cell Signaling and Interaction, Inflammatory Response</td>
</tr>
<tr>
<td>2</td>
<td>Cell Morphology, Carbohydrate Metabolism, Molecular Transport</td>
</tr>
<tr>
<td>3</td>
<td>Behavior, Cell Death and Survival, Hematological System Development and Function</td>
</tr>
<tr>
<td>4</td>
<td>Cell Death and Survival, Cellular Development, Hematological System Development and Function</td>
</tr>
<tr>
<td>5</td>
<td>Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry</td>
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<tr>
<td>6</td>
<td>Organ Morphology, Organismal Development, Reproductive System Development and Function</td>
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<tr>
<td>7</td>
<td>Cell Death and Survival, Cellular Growth and Proliferation</td>
</tr>
<tr>
<td>8</td>
<td>Gene Expression, Cancer, Organismal Injury and Abnormalities</td>
</tr>
<tr>
<td>9</td>
<td>Cellular Development, Cancer, Organismal Injury and Abnormalities</td>
</tr>
<tr>
<td>10</td>
<td>DNA Replication, Recombination, and Repair, Cell Cycle, Cellular Assembly and Organization</td>
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The evidence to date indicates that miRNAs can stably exist in body fluids, including saliva, urine, and blood thereby gate the potential differences in exosomal miRNA cargo for important consideration particularly in light of the disparate in subjects exposed to IH for 4 days. However, the relatively small concentrations of such exosomes precluded the separation of the exosomes into their various sources to study their independent functional effects on the endothelium. This is an important consideration particularly in light of the disparate and sometimes opposite effects of exosomes originating from different cell sources in plasma. Thus, future experiments using IH as the model paradigm should explore the potential differential contributions of each cell-derived exosome cluster to endothelial function.

Various nucleic acids have recently been identified in the exosomal lumen, including mRNAs and miRNAs. These exosomal RNAs can be taken up by either neighboring cells or by distant cells, and modulate recipient cell protein production and gene expression. Furthermore, exosomes can activate or inactivate different pathways on surrounding or remotely located cells, depending on their molecular composition, which is influenced by the activation state of the secreting cell. The assessment of biological functions of exosomes showed that they can deliver specifically their cargo. We chose to investigate the potential differences in exosomal miRNA cargo for several reasons. miRNAs are small (19–23 nucleotides) non-coding RNA molecules that bind to partially complementary mRNA sequences, resulting in target degradation or translation inhibition, and therefore are de facto ubiquitous regulators of cell function and survival in both health and disease. The evidence to date indicates that miRNAs can stably exist in body fluids, including saliva, urine, and blood thereby pointing to their unique value as prospective biomarkers. Here, we found that that the modifications of exosome cargo induced by IH led to consistent alterations in not only their cell source distribution, but also in their effects on naïve endothelial cells. We have to mention that other exosome cargos such as lipids and protein may have an effect on the disruption of endothelial cells. Indeed, D4 exosomes induced endothelial dysfunction as illustrated not only by disruption of a monolayer barrier, but also by the induction of adhesion and reduced endothelial nitric oxide synthase expression. Taken together, these findings illustrate that a component of the proatherogenic state characteristically present in most OSA patients may originate, at least in part, from the biological properties of exosomes released into the circulation during the episodic hypoxic events that recur throughout the night. In this context, the differences in monocyte adherence likely reflect the actual effects of exosome cargo on endothelium, although the possibility that some interactions between these murine innate immune cells and human epiplopes may take place cannot be excluded with complete certainty. Of note, the overall pathways identified in the bioinformatic analyses have been previously implicated either directly or indirectly in the pathophysiology of sleep apnea perturbations triggering vascular dysfunction.

The identification of mRNAs and miRNAs in exosomes and the ability of the transferred exosomal miRNA and mRNA to be translated in target cells constitute a major breakthrough in exosome biology. In particular, exosomal miRNAs are playing an important role in cell-to-cell communication and involved in a multitude of functions, both physiological and pathological processes. Among the 6 miRNAs differentially expressed in D4 versus D0 exosomes, has-mir-383-3p exhibits a known regulatory information network with 23 validated mRNA targets (Figure 8). One of the 23 genes in the RIN includes ARRB2 arrestin, beta 2, which is known to participate in agonist-mediated desensitization of G-protein-coupled receptors and cause specific dampening of cellular responses to stimuli such as hormones, neurotransmitters, or sensory signals. Likewise, the RNF5 ring finger protein 5 gene can regulate cell motility by targeting paxillin ubiquitination and altering the distribution and localization of paxillin in cytoplasm and cell focal adhesion, whereas the ZBTB7B zinc finger and BTB domain containing 7B encodes a zinc finger-containing transcription factor that acts as a key regulator of lineage commitment of immature T cell precursors as well as a transcriptional repressor of type I collagen genes. All these hierarchical functions have been previously shown to be affected in the context of OSA. However, rather than simply rely on known RINs and “theoretical targets,” we explored key interactions between exosomal cargo and target cell mRNAs by exposing naïve endothelial cells to D0, D4, and D8 exosomes and examining the conglomerate of affected mRNAs. Bioinformatic analyses revealed that 333 mRNA were significantly regulated by plasma exosomes in the context of IH, and provided insights into higher-order GO maps and KEGG pathways that are affected by IH, further reinforcing the concept that IH-induced alterations in circulating exosomes and their respective cargo elicits a downstream cascade of events that not only affects endothelial integrity, but also leads to a variety of endothelial cell perturbations that promote a coordinated...
induction of vascular dysfunction and atherosclerosis. Several reports suggest that miRNAs contained in exosomes can influence gene expression in target cells.\textsuperscript{12,13}

In conclusion, intermittent hypoxic exposures in otherwise healthy young adult subjects induce release of exosomes to the circulation that promote increased permeability and dysfunction of endothelial cells \textit{in vitro}. In this context, this work identified a unique and a select number of circulating exosomal miRNAs that may play important roles in the cardiovascular dysfunction associated with OSA, and may potentially assist in delineation of individually tailored mechanistically-based clinical treatment approaches of OSA patients in the near future. In addition, we also uncovered that IH-altered exosomal miRNAs regulate a unique set of endothelial mRNAs target genes, the latter clearly including well-established immune and atherosclerosis-related pathways.

REFERENCES


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