ONLINE SUPPLEMENT

Circulating extracellular vesicles in normotension restrains vasodilation in resistance arteries

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Short Title: Extracellular vesicles alter vascular tone

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Expanded Methods and Materials

EV morphology. Cryo-electron microscopy was performed to study the morphology of EVs[1-3]. An aliquot of 20 µL was applied to a glow-discharged, perforated carbon coated grid (2/2-4C C-flats), blotted with filter paper, and rapidly plunged into liquid ethane. The grids were stored in liquid nitrogen, and then maintained in the microscope using a Gatan 626 cryo stage. Images were recorded at a magnification of 29,000X on a FEI Tecnai F20 Twin transmission electron microscope operating at 120kV, with a nominal underfocus ranging from 3.5 to 5 mm and a pixel size of 0.388 nm at the specimen level. All images were recorded with a Gatan 4K x 4K pixel CCD camera.

EV size detection: Nanoparticle Tracking Analysis (NTA) was carried out using the ZetaView PMX 110 multiple parameter particle tracking analyzer (Particle Metrix, Meerbusch, Germany) configured with a 488 nm laser with a long wave-pass cut-off filter (500 nm) and a sensitive CMOS camera 640 x 480 pixels in size mode using ZetaView software version 8.02.28 provided from the manufacturer[4]. Pellet samples were diluted in 2 mL of 0.1 µm filtered (Minisart® high flow hydrophilic 0.1 µm syringe filter Sartorious) PBS to obtain a particle concentration between 1 x 10^7 and 1 x 10^8 particles/mL. The instrument was set to a constant temperature of 28°C, a sensitivity of 70, a shutter speed of 70 and a frame rate of 30 frames per second. Each sample was measured at 11 different positions throughout the cell, with 5 cycles of readings at each position in order to have a minimum of 1000 traces. Post-acquisition parameters were set to a minimum brightness of 30, a maximum size of 200 pixels, and a minimum size of 5 pixels. Automated cell quality control was checked using high quality deionized water. Camera alignment and focus optimization was performed using polystyrene 100 nm beads (Applied Microspheres, Leuden, Netherlands).

***Rat kidney microsomes enrichment (Mi)*:** A standard rough microsomal fraction was obtained by centrifugation of kidney homogenates for western blot analysis[5]. Kidneys obtained from 12 weeks old WKY were homogenized in 10 mL of 10 mM Hepes pH 7.4, 0.2 M Mannitol, 50 mM sucrose, 1 mM EDTA, 1X SIGMAFAST™ proteases inhibitor cocktail (Sigma-Aldrich code S8830) with a Potter-Elvehjem-type homogenizer at 4°C. The homogenate was centrifuged at 800 g for 10 min at 4°C in in a TX-400 Sorvall ST16R (ThermoFisher Scientific) swing bucket rotor (k Factor 9153). The supernatant was centrifuged at 20,000 g for 30 min at 4°C in an Eppendorf microcentrifuge 5424 (Eppendorf) fixed angle rotor. Finally, a standard rough microsomal fraction was obtained by centrifugation using Beckman Coulter XL-90 ultracentrifuge and a type 90 Ti Rotor (Beckman coulter) (k factor at maximum speed 25) at 116,000 g for 90 min at 4°C in polycarbonate tubes. Microsomal pellet (Mi) was resuspended either in 100 µL of PBS buffer or 100 µL electrophoresis solubilization buffer (ESB) for western blot analysis.

EV and non-EV protein analysis by western blotting. Protein assessment of isolated EVs and Mi (kidney microsomes) was performed[6]. 10 µg of protein was solubilized in ESB [made of 5% (w/v) sodium dodecyl sulfate (SDS), 40 mmol/L Tris-HCl pH 6.8, 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA), 20% (v/v) glycerol and 50 mmol/L dithiothreitol].

Samples were denatured overnight at room temperature. Proteins were separated by hand cast SDS-PAGE gradient gels (Resolving gel T= 5-20% (w/v); C=2.6%) in 25 mmol/L Tris, 192 mmol/L glycine and 0.1% (w/v) SDS buffer and transferred onto a 0.45 µm nitrocellulose membrane (Amersham™ Protean™ 0.45 µm NC, GE Heaelthcare) in a wet transfer system buffer made of 25 mmol/l Tris, 192mmol/L glycine and 20% (v/v) methanol. Nitrocellulose membranes were saturated with Odyssey blocking buffer (LI-COR Biosciences) and incubated in polyclonal rabbit anti-TSG 101 (Sigma, T5701-200UL) and rabbit anti-calreticulin (Novus Biologicals, NB600-101) over night at room temperature (23-24˚C) in Odyssey blocking buffer diluted 1:1 with PBS and 0.15% (v/v) Tween-20 at concentration of 1.0 µg/mL. After three 10min washes in PBS Tween (0.15%, v/v), membranes were incubated with anti-rabbit anti mouse dye-coupled secondary antibody 0.1 µg/mL (LI-COR Biosciences) in an Odyssey blocking solution diluted at 1:1 with PBS and 0.15% (v/v) Tween-10; 1 h at room temperature. Acquisition of the fluorescent signal was performed by Odyssey infrared imaging system with resolution set at 169 µm (LI-COR Biosciences).

EV protein analysis by flow cytometry. EV protein content was detected with imaging flow cytometry according to our recent publications[7, 8]. The following antibodies were mixed together (antibody mix): 5 µL CD105-fluorescein isothiocyanate (FITC) (Clone MEM-226, Product Code MA5-16954, Novus Antibodies), 5 µL CD31-phycoerythrin (PE) (Clone TDL-3A12, Product Code MA5-16954, ThermoFisher Scientific), 5 µL Annexin V (AV)- Alexa Fluor®568 (AF568), (Product Code A13202, ThermoFisher Scientific), 5 µL CD45- Alexa Fluor®405 (AF405) (Clone OX-1, Product Code NB100-64895AF405,Novus), and 5 µL CD42d-Allophycocyanin (APC) (Clone 1C2, Product Code 17-0421-82, eBioscience). The antibody mix was centrifuged at 21,000 g for 1 h. For controls, EVs were diluted into 100 µL of 1X AV buffer (Cat. 51-66121E; BD Biosciences) alone (EV Only) or 20 µL of the antibody mix was added to 100 µL of the 1X AV buffer (Buffer + Ab). To stain EVs, 20 µL of the antibody mix was added to EVs diluted in 100 µL of 1X AV buffer (EV + Ab). For compensation controls, 1 µL of each individual fluorescence dye conjugated antibodies (CD31-PE, CD105-FITC, ANN V–AF568, CD45-AF405, and CD42d-APC) was added to 100 µL of 1X AV buffer . The samples were then vortexed three times for 5 sec and then incubated for 45-60min in the dark at room temperature. After this time period, 1mL of 1X AV buffer was added to each sample and spun for 1 h at 21,000 g at room temperature. The supernatant was discarded and the pellet was resolubilized in 50 µL of the 1X AV buffer. Imaging flow cytometry [Amnis ImageStreamX Mark II (ISX)] was used to detect and determine the source and count of the EVs as described previously by our group[7, 8]. Briefly, the 60X objective was used to provide a pixel size of 0.3 μm^2. The excitation lasers were: 488 at 100 mW, 405 at 120 mW, 561 at 200 mW, 642 at 150 mW, and side scatter (SSC) at 70 mW. Several controls, indicated above and as previously described, were used to establish subpopulation gating. The acquired data were analyzed and EV counts were measured by a volumetric method provided by the software of ISX (IDEAS, Amnis Corporation; Application Version 6.2.64.0). FCS express6 DeNovo™ software was then used to create the histogram and dotplots. Quantum™ molecules of Soluble Fluorochrome (MESF) kit beads for FITC and PE (Bangs Laboratories Inc) were also used. The assignment of fluorescence intensity for each sample was extrapolated from the four (PE-R) and five (FITC) fluorescent

microsphere populations which carry well-known amounts of fluorescence dyes. After this calibration, MESF units were consequently assigned to each sample, thus guaranteeing a high measuring accuracy.

EV delipidation. EVs pellets were resuspended in 100 µL of 0.1 µm filtered PBS and delipidated by chloroform methanol[9]. Briefly, 400 µL of 100 % (v/v) methanol was added to the sample, vortexed and centrifuged for 10 seconds at 9000g. 200 µL of chloroform were added, vigorously vortexed and centrifuged for 10 seconds at 9000g. 300 µL of deionized water was added, mixed vigorously and centrifuged for 5 minutes at 9000g. The aqueous upper phase was discarded and the interface protein layer was precipitated by adding 300 µL of 100 % (v/v) methanol and centrifuged for 10 minutes at max speed and then resuspended in Krebs-HEPES buffer.

Liposomes. Liposomes were prepared by a modification of the Szoka/Papahadjopoulos filtration technique previously described[10]. Briefly, 40 mg DSPC (Avanti Plar lipids, Alabaster, AL), 20 mg cholesterol (Sigma Chemical Co, St. Louis, MO), 1 mg carboncyanine lipophilic dye DiI (Molecular Probes, Eugene, OR) (molar ratio of 1:47.3 DiI to DSPC) or DiO (Molecular Probes, Eugene, OR), and 4 mg PEG2000-DSPE (Avanti Plar lipids, Alabaster, AL) were dissolved in chloroform, placed in a glass vial and chloroform evaporated by the flow of argon gas while in a 37˚C bath. Traces of residual chloroform were removed by vacuum. Normal saline (10 mL) was then added and multilamellar liposomes were formed by mixing in a hot water bath. Resulting liposome were stored at -20˚C and counted by NTA.

Blood Pressure Measurements. Blood pressure on rats 7 weeks and older was measured with telemetry probes [Data Sciences International, (DSI)], which were inserted under sterile conditions into the descending aorta as previously described[11]. Briefly, animals were anesthetized using isoflurane. Using sterile procedure, a laparotomy was performed exposing the abdominal cavity and descending aorta. Ligatures were placed under the aorta, both distal and proximal to the renal artery and lifted to prevent blood flow. A catheter introducer pierced into the aortic artery and the telemetry probe catheter was inserted. Vetabond glue was used to fasten the catheter into place and prevent bleeding. The probe body was attached with interrupted sutures using 3-0 Prolene suture to the abdominal wall while closing the abdomen wall, and 4-0 Prolene suture was used for the skin closure. Mean arterial pressure (MAP) was recorded once weekly, specifically at 1:00 PM in the afternoon for 40 minutes. Due to the smaller size of 6 week old rats, blood pressure at 6 weeks of age was measured in WKY and SHR via direct carotid artery cannulation[11]. Under anesthesia (Inactin, Sigma), an incision was made along the right side of the trachea exposing the right carotid artery. A PE-50 tube was inserted into the right carotid artery and after ensuring blood flow, ligated into place with 4-0 silk ligature. The line was attached to a Digi-Med Blood Pressure Analyzer (Micro-Med, Inc) and MAP was recorded.

Pressure Myography. Freshly isolated 3rd or 4th order rat or mouse mesenteric arteries (< 220 µm) were placed into ice-cold Krebs-HEPES buffer (containing (in mM) NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 4, KH_2PO_4 1.2, $CaCl_2$ 2, Hepes 10, glucose 6; pH

7.40). The vessels were then mounted in a pressure arteriograph [Culture Myograph system – 202CM; Danish MyoTechnology (DMT)] with the lumen filled with Krebs-HEPES buffer as previously described[12]. The vessels were equilibrated for 30 min at 80 mmHg and 37˚C. The lumen and bath solutions were then replaced with EV containing Krebs-HEPES solution while control vessels were perfused with regular buffer. 30 µL of the isolated EV solution (or liposomes) were placed into 3 mL buffer for the lumen solution and 70 µL of EVs were placed into the 10 mL circulating bath. The vessels were then equilibrated, at 80 mmHg and 37˚C, for an additional 10 min. Following pre-constriction with 10 µM phenylephrine (PE) for 10 minutes to obtain a stable constriction, increasing concentrations of ACh were added into the bath to examine the endothelial-dependent vasodilation of these vessels. Full ACh curves were obtained for each vessel under a given treatment condition. Vessels were used from at least 4 different rats per treatment group. When L-nitro-arginine methyl ester (L-NAME) was used in experiments, 100 µM L-NAME (dissolved in dH_2O) was added to the bath after the first 10 min of the initial equilibration to allow for a 30 min total incubation. Endothelial cell health was confirmed by obtaining >80% vasodilation to NS309 (small- and intermediate-conductance calcium-activated potassium (SK_{Ca}/IK_{Ca}) channels) and smooth muscle cell health confirmed by constriction >50% to PE. The vessels were then washed with a Ca^{2+}-free Krebs-HEPES solution supplemented with 1 mM Ethyleneglycol-O,O′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid and 10 µM sodium nitroprusside to obtain maximal passive diameter of the vessels. Internal diameter was measured at each step using the DMT MyoVIEW software. Vasoconstriction to PE was calculated as percent of initial diameter: % initial diameter = D_{PE} / $D_{initial}$ * 100. Vasodilation to ACh or NS309 was calculated as a % relaxation: % relaxation = [(D_{ACh} – D_{PE}) * 100]/(D_{max} – D_{PE}), where D_{PE} was the diameter of the artery 10 minutes after application of 10 µM PE, $D_{initial}$ was the diameter prior to the addition of PE, D_{ACh} was the diameter after application of a given dose of ACh, and D_{max} was the maximal diameter measured at the end of experiment. Each vessel underwent only one experimental condition and one full ACh dilation curve as the long-term or lingering effect of EVs after treatment in an artery is unknown. Data were replotted in multiple figures (such as control WKY arteries) because comparisons were able to be done across all groups as all treatment groups underwent the same experimental design. For statistical comparisons, groups were compared that were plotted within the same graph.

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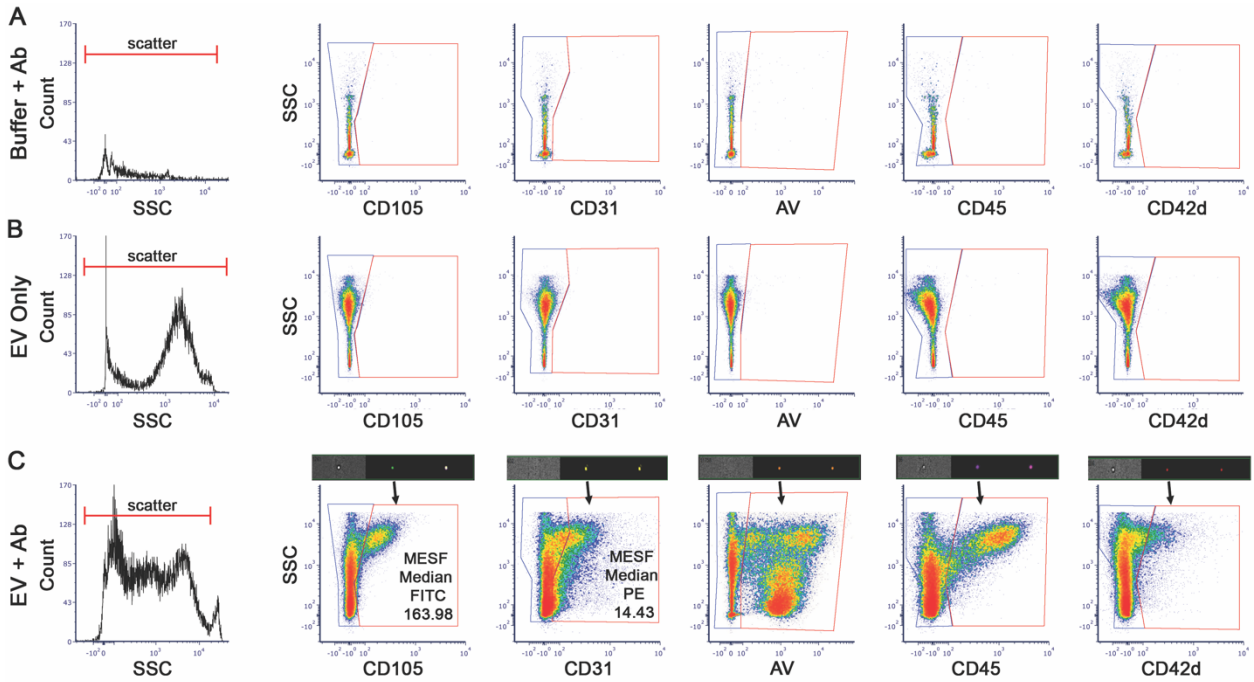
Supplemental Table S1. Summary data for vasoreactivity experiments on WKY arteries (mean ± s.d.). One-way ANOVA (vs. 12 wk WKY Arteries Control; * p<0.05; ** p< 0.01).

	Resting Diameter (μm)	Passive Diameter	Max PE Constric-tion Diameter (μm)	Max % PE Constric-tion	Max ACh Dilation Diameter (μm)	Max % ACh Relaxation	EC_{50} ([Ach], M)
12 wk WKY Arteries Control	230.0 ± 25.22	242.4 ± 26.07	54.48 ± 9.72	23.95 ± 3.83	224.70 ± 34.85	90.50 ± 11.47	$1.70x10^{-7}$ ± $1.50x10^{-7}$
12 wk WKY Arteries + L-NAME	207.5 ± 24.79	253.9 ± 19.51	57.79 ± 14.23	28.65 ± 8.43	154.60 ± 61.00 p=0.05	49.06 ± 26.89 **	$3.70x10^{-7}$ ± $3.03x10^{-7}$
12 wk WKY Arteries + 12 wk WKY EV	141.0 ± 53.29	197.6 ± 26.13	43.75 ± 17.30	34.53 ± 19.02	151.40 ± 35.94 **	68.55 ± 19.09 p=0.05	$3.22x10^{-7}$ ± $1.49x10^{-7}$
12 wk WKY Arteries + 12 wk SHR EV	151.8 ± 37.81	201.6 ± 14.86	42.19 ± 6.60	28.51 ± 5.72	186.00 ± 24.41	89.95 ± 6.77	$1.27x10^{-7}$ ± $3.85x10^{-7}$
12 wk WKY Arteries + L-NAME + 12 wk WKY EV	139.3 ± 19.01	213.3 ± 28.74	50.99 ± 12.42	38.19 ± 8.43	136.70 ± 8.68 *	53.87 ± 6.89 *	$6.55x10^{-7}$ ± $7.96x10^{-7}$
12 wk WKY Arteries + L-NAME + 12 wk SHR EV	139.2 ± 29.68	217.8 ± 5.55	51.95 ± 10.78	40.33 ± 17.79	164.00 ± 37.60 p=0.10	68.75 ± 22.35	$3.58x10^{-7}$ ± $4.06x10^{-7}$
12 wk WKY Arteries + 6 wk WKY EV	160.9 ± 42.82	218.6 ± 11.32	59.02 ± 42.62	35.05 ± 13.81	163.20 ± 49.69 p=0.09	68.38 ± 22.30 p=0.08	$1.69x10^{-7}$ ± $1.46x10^{-7}$
12 wk WKY Arteries + 6 wk SHR EV	166.3 ± 46.06	208.6 ± 17.87	45.87 ± 5.65	29.73 ± 8.47	145.20 ± 40.19 *	61.97 ± 25.15 p=0.11	$2.97x10^{-7}$ ± $4.13x10^{-7}$
12 wk WKY Arteries + 12 wk WKY Delipidated EV	121.9 ± 23.21	189.8 ± 29.85	36.00 ± 3.99	30.04 ± 4.46	123.30 ± 13.80 **	58.44 ± 14.28 *	$2.53x10^{-7}$ ± $1.58x10^{-7}$
12 wk WKY Arteries + 12 wk SHR Delipidated EV	153.4 ± 33.91	212.6 ± 27.11	42.91 ± 5.57	29.44 ± 9.09	147.00 ± 41.18 **	61.71 ± 19.05 *	$2.87x10^{-7}$ ± $7.75x10^{-7}$
12 wk WKY Arteries + Liposomes	137.3 ± 26.96	213.5 ± 22.13	54.87 ± 9.28	42.45 ± 16.79	182.50 ± 8.87	81.29 ± 6.66	$2.79x10^{-7}$ ± $2.19x10^{-7}$

Supplemental Table S2. Summary data for vasoreactivity experiments on SHR arteries (mean ± s.d.). One-way ANOVA (vs. 12 wk SHR Arteries Control).

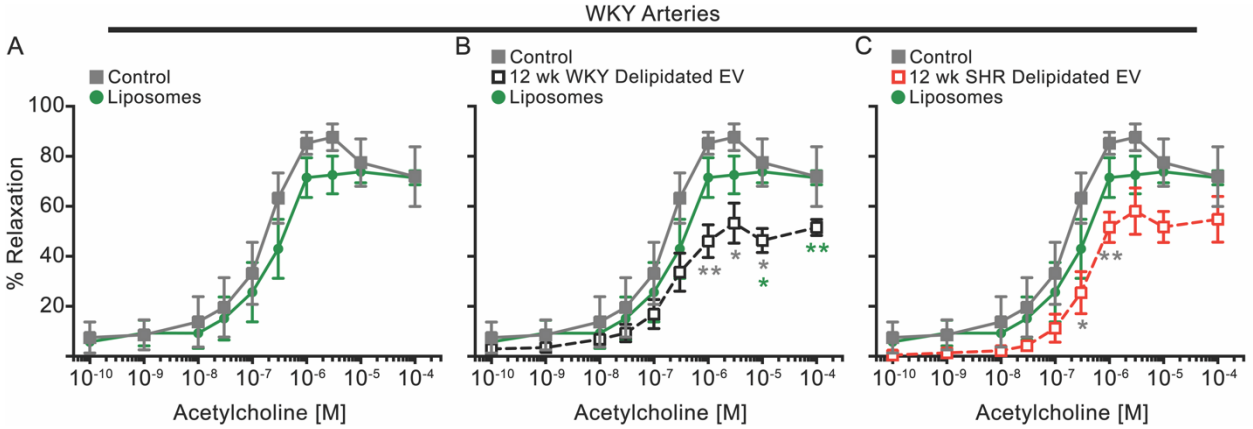
	Resting Diameter (μm)	**Passive Diameter**	**Max PE Diameter (μm)**	**Max % PE**	**Max ACh Diameter (μm)**	**Max % ACh**	**EC_{50} ([Ach], M)**
12 wk SHR Arteries Control	169.3 ± 44.54	196.4 ± 32.97	24.28 ± 7.75	15.57 ± 7.29	179.90 ± 38.91	89.76 ± 10.03	$2.14x10^{-7}$ ± $1.31x10^{-7}$
12 wk SHR Arteries + L-NAME	164.6 ± 27.08	196.2 ± 23.97	21.62 ± 14.87	13.03 ± 9.10	147.30 ± 35.58	72.71 ± 22.50	$1.06x10^{-6}$ ± $1.25x10^{-6}$
12 wk SHR Arteries + 12 wk WKY EV	135.9 ± 20.87	191.2 ± 17.70	20.12 ± 6.08	15.28 ± 5.67	166.50 ± 29.45	84.98 ± 12.90	$2.85x10^{-7}$ ± $4.99x10^{-7}$
12 wk SHR Arteries + 12 wk SHR EV	143.4 ± 61.52	190.4 ± 40.68	26.16 ± 10.59	22.49 ± 13.22	165.30 ± 51.71	83.24 ± 14.83	$2.61x10^{-7}$ ± $3.01x10^{-7}$

Supplemental Figure S1



Supplemental Figure S1: Imaging flow cytometry phenotyping of circulating EVs. EVs isolated from rat plasma was used for quantification of endothelial, leukocyte, platelets markers. Histograms (left) were used to identify EV population using side scatter (SSC) and then gated for each marker using compensation controls (right). Buffer+Ab control (A) and EV only control (B) were used to set gates appropriately. An example of EVs stained with antibody mixture (EV + Ab) is provided (C). Imaging flow cytometry for EV detection combines an increased fluorescence sensitivity and image confirmation ability ensuring EV detection at least down to 100nm in size[7]. In addition, FITC & PE MESF measurements are also reported in this figure (C) demonstrating a typical MESF range for EVs[7].

Supplemental Figure S2



Supplemental Figure S2: Treatment with liposomes does not reduce ACh-mediated vasodilation in WKY arteries. Arteries isolated from WKY were exposed to liposomes (A), delipidated WKY EVs (B), or delipidated SHR EVs (C). ACh-mediated vasodilation was then evaluated. Data presented as mean ± sem. (Control: n=6; 12 wk WKY delipidated EV, 12 wk SHR delipidated EV, and Liposomes: n=4) Mixed-effects model for repeated measures with Sidak's multiple comparisons test (Control vs 12 wk WKY Delipidated EV or 12 wk SHR Delipidated EV: grey; Liposomes vs 12 wk WKY Delipidated EV or 12 wk SHR Delipidated EV: green): * $p < 0.05$, ** $p < 0.01$.