SUPPLEMENTAL MATERIAL

Endothelial mineralocorticoid receptor mediates diet induced aortic stiffness in females

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Expanded Methods:

Aortic stiffness by pulse wave velocity *in vivo*

Determination of PWV is based on the transit time method utilized to determine the difference in arrival times of a Doppler pulse wave at two locations along the aorta a known distance apart. Each of the pulse wave arrival times is measured as the time from the peak of the ECG R-wave to the leading foot of the pulse wave at which time velocity begins to rise at the start of systole. The distance between the two locations along the aorta is divided by the difference in arrival times and is expressed in m/s. Velocity waveforms were acquired at the aortic arch followed immediately by measurement at the descending aorta 35 mm distal to the aortic arch. Ultrasound procedures were performed on isoflurane-anesthetized mice (1.75% in 100% oxygen streams).

AFM Imaging and force measurement

A MFP-3D AFM 89 (Asylum Research Inc. Goleta, CA) mounted on an Olympus IX81 microscope (Olympus Inc.) was used for biomechanical measurements and estimate elastic modulus/stiffness. AFM measurements were conducted at room temperature (~25^{0}C). For stiffness measurements, an AFM cantilever (MLCT, 92 Bruker-nano, Goleta, CA) was used to perform repeated cycles of nano-indentation and retraction cycles on the cell surface. The parameters employed were 0.3 Hz sampling frequency, with an approach/retraction velocity of 960 nm•sec-1, 1600 nm traveling distance for one sampling cycle (indentation and retraction), and approximately 400-600pN loading force. Force curves were generated over a period of two minutes and analyzed using NForceR software (registration number TXu1-328-97 659) and MATLAB. The mean of these elastic modulus (i.e., stiffness) values was computed for each indentation site and then averaged together for each group. E-99 modulus were obtained using a length of 100-300 nm of the AFM indentation curve, after the initial point of contact that was fit with a Hertz model as shown in equation:

$$F = \frac{2}{\pi}\frac{E}{(1-\nu^2)}\frac{\delta^2}{\tan\alpha}$$

Where, *E* is the *E*-modulus, *F* is the force exerted by AFM probe on tissue surface, *δ* is indentation depth into the sample, *a* is the half-opening angle of the AFM tip, and *v* is the Poisson ratio. The tissues were considered as a gel and the Poisson ratio *v* was assumed at 0.5. To obtain topographical images of EC, the AFM was operated in contact mode. The area of the tissue surface that was scanned in these experiments was 40 x 40 μm and the digital density of the scanned area was 512 × 512 pixels. Stylus type AFM probes (Model: MLCT-C, k = 15 pN/nm, Bruker, Santa Barbara, CA) were used to perform surface 108 scanning at 0.4 Hz frequency with approximately 300-500 pN tracking force.

***Ex vivo* aortic activity and flow-induced dilation**

A 2 mm segment of thoracic aorta was collected immediately after euthanasia and placed in ice-cold physiological salt solution (PSS) containing (in mM): 119 NaCl, 4.7

KCl, 2.5 Cacl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 0.027 EDTA, 5.5 glucose, and 25 $NaHCO_3$, pH 7.4. Aortic contractile state was ascertained by KCl (80 mM•L^{-1}). Aortas were preconstricted with U46619 (100 nM). Dilation of arterial rings to acetylcholine (1 nm to 10 mM), the NO-donor sodium nitroprusside (1 nM to 10 mM), and to insulin ((Novolin R, Novo Nordisk; 0.1 to 300 ng•ml-1) was assessed by cumulative addition of agonist to the vessel bath. The doses of insulin utilized represent physiological (fasting ~0.2 ng•ml-1; post-prandial ~2 ng•ml-1), pathophysiological (>5 ng•ml-1) and pharmacological (>10 ng•ml-1) levels. At the end of each experiment, the PSS bath solution was replaced with Ca^{2+}-free PSS to determine maximal passive force. Aortic dilator responses are presented as percent maximal relaxation, calculated as [(Fb-Fd)/(Fb-Fmin)]*100, where Fd is force after a drug intervention, Fb is baseline force, and Fmin is maximal passive force.

Mesenteric resistance arteries were bathed in albumin-free physiological saline solution (PSS) containing (in mM) 145.0 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.0 $MgSO_4$, 1.2 NaH_2PO_4, 0.02 EDTA, 2.0 Pyruvic Acid, 5.0 Glucose and 3.0 MOPS buffer, and perfused with PSS containing 0.15 Albumin, pH of 7.4. Throughout the experiment, chambers were mounted on inverted microscopes with CCD cameras. Luminal diameter and wall thicknesses were recorded using a video caliper (Living Systems Instrumentation, Burlington, VT, USA) and a Powerlab data acquisition system (ADInstruments Inc, Colorado Springs, CO, USA). To test for viability, the cannulated arteries were allowed to stabilize for 40 min and then exposed to PSS in which NaCl was substituted equimolarly with 80 mM KCl. Only arteries that constricted more than 20% to this 80 mM K^+ solution were used in the analyses. After the exposure to high K^+, the arteries washed three times with fresh PSS and allowed to stabilize for 10 min. Vessels were subsequently pre-constricted with 10^{-5} M phenylephrine for 5 min and then exposed to eight increasing steps in flow ratios (0 to 26 µl/min) to determine flow (shear stress)-induced endothelium-mediated vasodilation. The changes in flow were induced with a peristaltic pump placed in the inflow pipette, while a pump in the outflow pipette was used to maintain intravascular pressure constant at 70 mmHg. Vessels remained at each flow ratio for 5 min until internal diameter became stable. Subsequently, flow was interrupted and the vessels washed three times as before. Amiloride (1 µM) was then added both intra- and extraluminally to the vessel. After a 20-min incubation in amiloride, the arteries where pre-constricted and exposed to increasing flow ratios as before, while in the presence of amiloride. At the end of each experiment flow was stopped and the arteries exposed to Ca^{2+}-free PSS with 2 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and 10^{-4} M adenosine for 10 min to obtain maximal passive diameter.

Measurement of ex vivo production of NO

Ex vivo production of NO in aortic explant was measured by using 4-amino-5-methylamino-2,7-difluorofluorescein (DAF-FM) diacetate (Molecular Probes, Eugene). Aortic explants were incubated with 5 µM DAF-FM diacetate for 30 min in DMEM medium at 37℃. After washing the aortic explants in DMEM medium for removing the excess dye, aortic explants were exposed to 10 µM acetylcholine for 10

min. Intracellular NO levels were determined from the fluorescence intensity of DAF-FM *via* confocal microscopy.

Chromatin immunoprecipitation (ChIP) and quantitative RT-PCR

ChIP analysis was carried out with the Imprint ChIP kit (Sigma, St. Louis, MO, USA). To release the chromatin, 20 mg of aortic tissue was homogenized and cross-linked with 1% formaldehyde. This was then digested with micrococcal nuclease (2 U/ml; Sigma) to provide ~500 bp of fragmented genomic DNA. The extract was either aliquoted as genomic input DNA or immunoprecipitated using MR antibody (Cell Signaling Technology, Danvers, MA). Following hydrolyzation of the crosslinks, the DNA was collected and quantitative PCR were performed on both genomic input and ChIP DNA. PCR primers were designed to flank the two MR consensus sequences in the ENaCα promoter. Quantitative PCR of the ChIP products and genomic input DNA was performed by real-time PCR using SYBR green (Bio-Rad, Hercules, and CA). The amount of ChIP DNA present in each sample was reported as percentage of genomic input DNA.

For qPCR, total RNA was isolated from thoracic aorta extracts using TRIzol reagent (Sigma, St Louis). RNA yield was determined using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). First-strand cDNA synthesis was done using 1 µg total RNA with oligo dT (1 µg), 5× reaction buffer, $MgCl_2$, dNTP mix, RNAse inhibitor, and Improm II reverse transcriptase as per Improm II reverse transcription kit (Promega, Madison, WI). After the first strand synthesis, real-time PCR was done using 8 µl cDNA, 10 µl SYBR green PCR master mix (Bio-Rad Laboratories) and forward and reverse primers (10 pM/µl) (Integrated DNA Technologies, San Diego, CA) using a real-time PCR system (CFX96; Bio-Rad Laboratories). The primer sequences used were: MR, Forward: 5'-CAGCTAGCTTTGGCAGTTTC-3', Reverse: 5'-GTGTGACCTTGAGCCTCTATT-3'; ENaCα, Forward: 5'- AGCACAACCGCATGAAGA-3', Reverse: 5'- CCTCGAACAGCAAAGCAAAC-3'; MCP-1, Forward: 5'-GATGCAGTTAATGCCCCACT-3'; Reverse: 5'-TTCCTTATTGGGGTCAGCAC-3'; IL17, Forward: 5'-CTTCACCTTGGACTCTGAGC-3'; Reverse: 5'-TGGCGGACAATAGAGGAAAC-3'; CD86, Forward:5'- GACCGTTGTGTGTGTTCTGG-3', Reverse:5'- GATGAGCAGCATCACAAGGA-3'; CD11c, Forward: 5'-ATGAAGAACCTCCGGGAAAT -3', Reverse:5'- GCTTAGATCATGGCGTGGTT -3'; CD11c, Forward: 5′-ACACAGTGTGCTCCAGTATGA-3′, Reverse:5′-GCCCAGGGATATGTTCACAGC-3′; IL10, Forward:5'- CCAAGCCTTATCGGAAATGA-3', Reverse:5'- TTTTCACAGGGGAGAAATCG-3'; CD206, Forward:5'-CAAGGAAGGTTGGCATTTGT-3', Reverse:5'- CCTTTCAGTCCTTTGCAAGC-3'; GAPDH, Forward: 5'-GGAGAAACCTGCCAAGTATGA-3', Reverse: 5'-TCCTCAGTGTAGCCCAAGA-3'. The specificity of the primers was analyzed by running a melting curve. The PCR cycling conditions used were 5 min at 95°C for initial denaturation, 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C. Each real-time PCR was carried out using three individual samples in triplicates, and the threshold cycle values were averaged. Calculations of relative normalized gene expression were done using the Bio-Rad CFX manager software based on the ΔCt method. The results were normalized against housekeeping gene GAPDH.

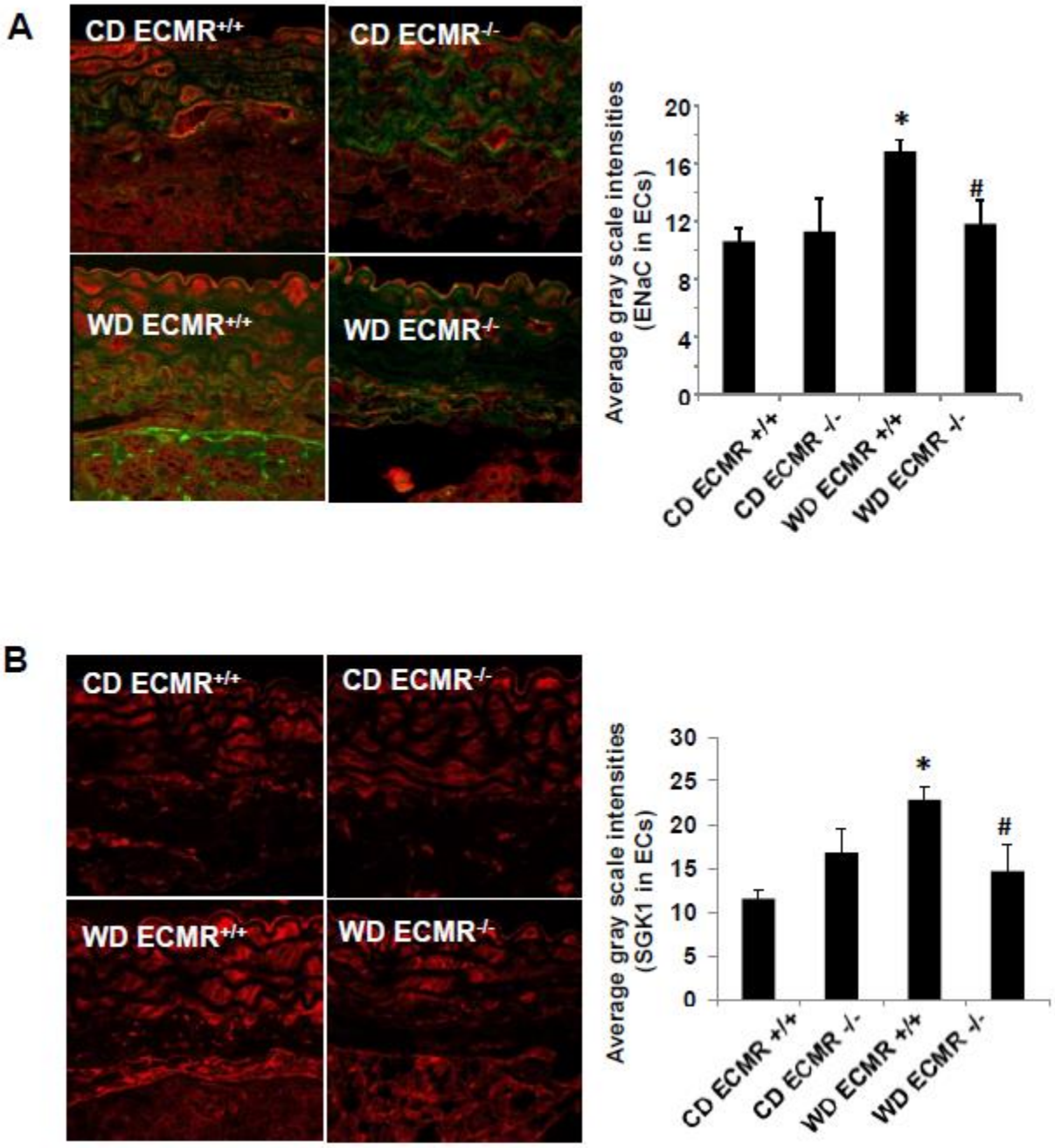
Western blot

Aortic tissues were collected and lysed in lysis buffer and the protein concentration of the lysate was determined by Bio-Rad protein assay. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Non-specific proteins were blocked by incubation in blocking buffer and the membranes were incubated overnight at 4°C with blocking buffer containing antibodies to p-ERK1/2 Thr202/Tyr204, Erk 1/2, p-Akt Serine473, Akt (Cell Signaling Technology, Danvers, MA) and p-eNOS Serine1177 and eNOS (BD Biosciences, San Jose, CA).

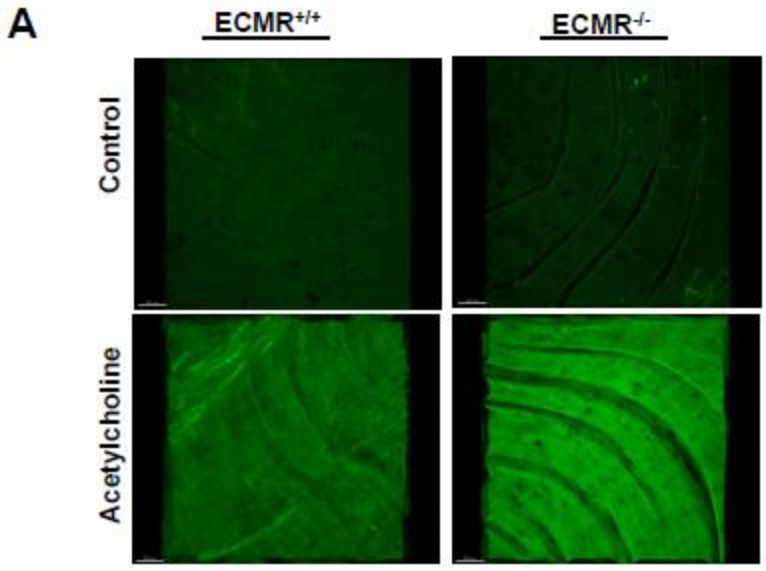
Vascular Remodeling

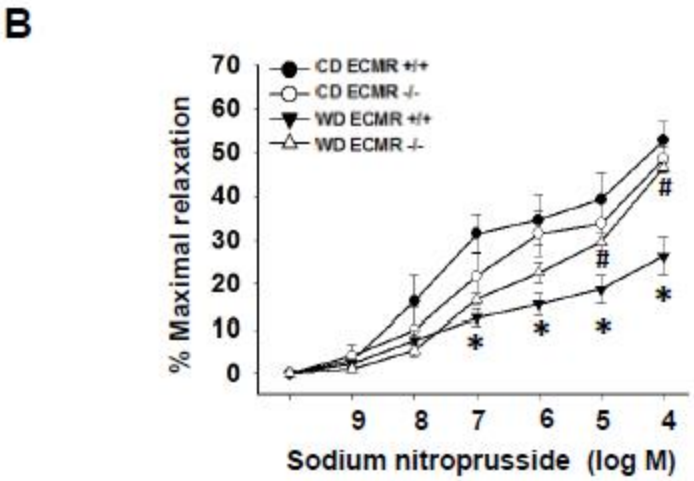
A 2 mm segment of thoracic aorta was fixed in 3% paraformaldehyde, dehydrated in ethanol, paraffin embedded, and transversely sectioned in 5μm slices. Four sections each for 4-5 mice per group were examined. To evaluate aortic fibrosis sections were stained with picrosirius red (PR) stain for the determination of collagen accumulation. The areas and the intensities of red color on the images which were stained with picro-Sirius red which are the indicative of collagen deposition were quantified as gray scale intensities by using MetaVue software. Fluorescent immunohistochemistry was used to quantify ENaC, SGK1, osteopontin, and Nox4 expression. Aorta samples of were prepared as described above. Five μm sections were dewaxed, rehydrated, and placed in 95ºC citrate buffer for 25 minutes for antigen retrieval. Non-specific binding cites were blocked with 5% BSA and 5% donkey serum. Next, sections were incubated with antibody to ENaCα, SGK1, osteopontin, and Nox4 expression (Cell Signaling Technology, Danvers, MA) overnight at room temperature. After several washes the sections were incubated with appropriate secondary antibodies, mounted with Mowiol and the section were checked under a bi-photon confocal microscope (Ziess). The areas and the intensities of red color were quantified by using MetaVue.

Supplemental Figures and Figure Legends

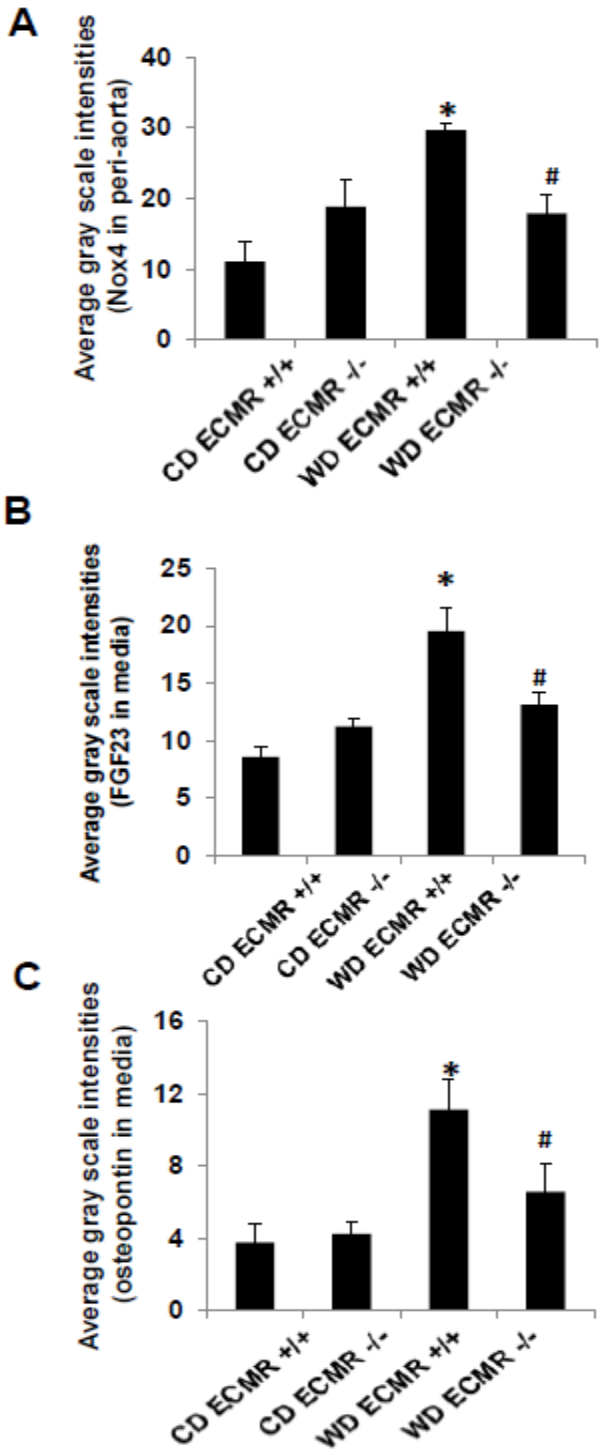


Online Figure I: The higher magnification images in the expression of ENaC (A) and SGK1 (B) in aortic endothelium with corresponding measures of average gray scale intensities. Scale bar = 50 μm. n=4 to 5 per group. **P*<0.01 compared with CD ECMR$^{+/+}$; # *P*<0.05 compared with WD ECMR$^{+/+}$.

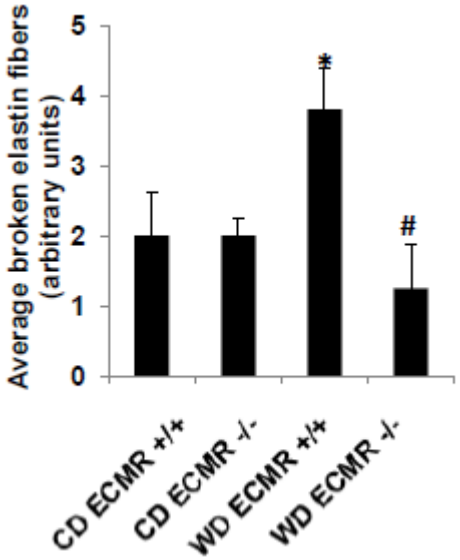




Online Figure II: (A) Acetylcholine-induced bioavailable NO is greater in aortic explants of ECMR$^{-/-}$ *ex vivo*. n=2 per group. (B) Vasodilator responses of isolated aortic rings to the endothelium-independent vasodilator, sodium nitroprusside. n=4 to 5 per group. *P<0.05 compared with CD ECMR$^{+/+}$; # P<0.05 compared with WD ECMR$^{+/+}$.



Online Figure III: Western diet induced Nox4, FGF23, and osteopontin is prevented in ECMR$^{-/-}$ mice. Representative images immunostaining for Nox4 (A), FGF23 (B), and osteopontin (C) with corresponding measures of average gray scale intensities below. n=5-6 per group. Scale bar = 50 μm. . *P<0.05 compared with CD ECMR$^{+/+}$; # P<0.05 compared with WD ECMR$^{+/+}$.



Online Figure IV: Western diet induced the broken elastin fibers are prevented in ECMR$^{-/-}$ mice. Quantified the broken elastin fibers were quantified in in each 40X bright-field images. n=5-6 per group. *P<0.05 compared with CD ECMR$^{+/+}$; # P<0.05 compared with WD ECMR$^{+/+}$.