Supplemental Material for:

Mineralocorticoid and Estrogen Receptors in Endothelial Cells Coordinately Regulate Microvascular Function in Obese Female Mice

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Supplemental Methods:

Investigators were blinded to genotype during in vivo and ex vivo experiments and blinded to all groups for the expression and in vitro studies.

Mice

All animal studies were approved by Tufts University Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. EC-MR-KO and EC-ERα/MR-KO mice were generated by crossing the respective floxed receptor mice with the constitutively active VE-Cadherin-Cre (Cdh-Cre). Six to eight week old female EC-MR-KO, EC-ERα/MR-KO and their floxed receptor, Cre negative littermates on C57Bl/6 background were randomized to normal chow diet (lean; Envigo 2918) or high fat diet (obese; Envigo; TD.88137) for 20 weeks. Mice were housed in 12 hour light and 12 hour dark cycles at a temperature of 68-72°F with free access to food and water. Mice were euthanized by terminal tissue harvest under isoflurane anesthesia.

Fasting Glucose Measurement

After 20 weeks on diet, mice were weighed and then fasted four hours and fasting blood glucose was measured using tail snip and glucose strips (True Balance).

Plasma Aldosterone Measurement

After 20 weeks on diet and immediately prior to sacrifice, facial vein blood was collected in BD Microtainer Lavender tubes and centrifuged at 2000g for 20 minutes at 4°C. Plasma was removed and frozen until measurement. Aldosterone was measured by radioimmunoassay (Tecan, MG13051) according to manufacturer's instructions.

Genotyping, Gene Recombination and Primers

Mouse genotype was confirmed by Transnetyx for each floxed and Cre allele in all mouse colonies.Endothelial cell-specific recombination of MR and ERα in Cdh-Cre x $MR^{fl/fl}$ and Cdh-Cre x $MR^{fl/fl}$ x $ERα^{fl/fl}$ was confirmed by endpoint PCR. Mice were perfused with normal saline via the left ventricle and the lung was harvested. Genomic DNA was extracted from lung tissue using the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions. The primer sequences below and the Platinum PCR Supermix (Invitrogen) were used to amplify the floxed exon 5 and 6 region of the *Nr3c2* gene encoding the MR or the floxed exon 3 region of the *Esr1* gene encoding ERα. PCR products were separated on a 2% agarose gel. The expected amplicon sizes of the WT, floxed, and recombined MR alleles are 314, 364, and 454 base pairs, respectively. The expected amplicon sizes of the WT, floxed, and recombined ERα alleles are 310, 344, and 551 base pairs, respectively.

MR_F: 5'-CCA CTT GTA TCG GCA ATA CAG TTT AGT GTC-3'
MR_R1: 5'-CAC ATT GCA TGG GGA CAA CTG ACT TC-3'
MR_R2: 5'-GAT TGT CAG GAA AAC ACA TTG AGC AGC-3'

ERa_F: 5'-GGG TCA TCA CTG GGT CTG TGT TC-3'
ERa_R1: 5'-CTT CCA TTG TCT CTT TCT GAC ACA TGC-3'
ERa_R2: 5'-CAG AGT CAG TCT GGA GTA AAG ATC AGA AC-3'

Vascular RNA extraction and qPCR

Mesenteric arcades were harvested, washed in saline and placed into RNA Later for future dissection. Arteries were dissected free of fat and all of the arterial branches from two mice were pooled**.** ESR1 forward primer sequence CCTCCCGCCTTCTACAGGT. ESR1 reverse primer sequence CACACGGCACAGTAGCGAG. Gene expression was normalized to beta 2 microglobin (B2M forward primer sequence: GCTATCCAGAAAACCCCTCAA) B2M reverse primer sequence:CATGTCTCGATCCCAGTAGACGGT) and mRNA expression quantified by the $\Delta\Delta$CT method.

Vascular Function

Arteries were harvested and maintained in physiological salt solution (in mmol/L: 130 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 0.03 EDTA, 1.6 $CaCl_2$, 14.9 $NaHCO_3$, 1.18 KH_2PO_4, and 5.5 glucose). Internal circumference (IC) of the artery at 100 mmHg was determined and the arteries were stretched to 0.9 IC. Physiological salt solution with calcium was made fresh daily and continuously bubbled with 95% oxygen and 5% carbon dioxide. Smooth muscle cell function was confirmed with >1 mN constriction to 3 μM phenylephrine and endothelial function was confirmed with >50% dilation to acetylcholine (Ach, 1μM, Sigma) or NS309 (1μM, Tocris). Inhibitors were added to the bath at 0.1% or less of the bath volume. Data is presented as percent relaxation [((mN force at ACH concentration- mN force at 3μM PE preconstriction)/(mN force at 3μM PE – mN force at pre-PE baseline)*100]. Difference in the area under the curve (dAUC) was calculated by quantifying the area under the curve for arteries with A: dilation to ACh and B: dilation to ACh in the presence of LNAME and then subtracting B from A.

Pharmacological agents

Vascular function drugs include: ACh, phenylephrine, L-N^G-Nitro arginine methyl ester (LNAME, 100 μM, Sigma), ICI 182, 780 (1 μM, Tocris) 1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride (MPP; Tocris 100 nM) and PHTPP 100nM (Tocris 100 nM). All vascular function drugs were dissolved in DMSO except LNAME (distilled water). For in vitro studies, aldosterone (Aldo, 10 nM, Sigma) was dissolved in DMSO and estradiol (E2) was dissolved in ethanol (10 nM, Sigma). Appropriate vehicle was added to control cells or vessels for each experiment.

Cell Lines and Cell Culture:

Stable expression of ERα in EA.hy926 cells

EA.hy926 cells (a human umbilical vein endothelial cell hybrid) stably expressing ERα were generated previously. The expression of functional ERα was previously confirmed by ERα immunoblotting and ERE-luciferase reporter assays.[1]

MR knockdown in EAhy926 cells expressing stable ERα

EA.hy926 ERα stable expressing cells were grown in DMEM with 10% bovine growth serum (BGS) to 60% confluency. Then media was removed and replaced with Opti-MEM Reduced Serum Medium. Cells were transfected with siRNA targeting MR or scramble oligo as negative control (Integrated DNA Tech) using 15 pmol RNAi/control oligo duplex mixed with 5 µl Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol and cells cultured for 48 hours. MR knockdown was confirmed by immunoblotting. The EA.hy926 ERα stable expressing line +/- MR siRNA were grown in DME phenol red free medium with 10% stripped-BGS for 24 hours, switched to serum free medium for 24 hours, and then cells were treated with aldosterone (10 nM) +/- E2 (10 nM) for 20 min before harvest for immunoblotting. Vehicle-treated cells as well as all treatment groups received an equivalent amount of both DMSO and ethanol.

HEK293 Cell Transfection
HEK293 cells (American Type Culture Collection (ATCC)) were maintained in phenol red-containing DMEM (Gibco) with 10% fetal bovine serum (FBS, Atlantic Biologicals). HEK293 cells were transiently transfected with MR or ERα full length or peptide expression plasmids by PolyFect transfection reagent (Qiagen). The MR expression plasmid contains the full length human MR cDNA in the CMX expression vector with an N-terminal hemagluttanin (HA) tag. The human full-length ERα is expressed in the pCDNA 3.1 vector. The ERα sequence coding amino acids 176-253, which disrupts striatin interacting with ERα, is in the pEGFP-C2 vector (Clontech), as described.[1] Empty vector controls are included in all studies. Prior to harvest for immunoblotting, HEK cells were grown in phenol red free medium with 10% stripped-BGS for 24 hours, switched to serum free medium for 24 hours, and then cells were treated with aldosterone (10 nM) +/- E2 (10 nM) for 20 min.

Spironolactone inhibition of EC
Human umbilical vein endothelial cells (HUVEC), isolated from umbilical vein tissue in our lab, (passage 4 to 6) were cultured in M199 medium (Gibco, Gaithersburg, MD**)** supplemented with 10% bovine growth serum **(**GE Healthcare Life Sciences), 0.1 mg/mL heparin (Sigma, St. Louis, MO), and 50 µg/mL endothelial cell growth supplement (Biomedical Technologies) at 37°C, 5% CO_2. HUVEC were treated with 1µM Spironolactone or Vehicle (DMSO) for 20 hours, harvested and cell lysates were immunoblotted for ER alpha and GAPDH.

Immunoprecipitation
Cells were harvested in lysis buffer (20 mM Tris-Cl, pH 7.5, 0.137 M NaCl, 2 mm EDTA, pH 7.4, 1% Triton, 10% glycerol, 25 mM glycerol phosphate, and in the presence of phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors). Cell lysate was incubated with 4 micrograms of non-immune rabbit IgG or rabbit anti-striatin (abcam, ab193000)) antibody overnight at 4°C. Protein G beads (abcam) were washed with dH2O and PBS twice, then incubated with lysate and antibody at 4°C for 2 hours. The pellets obtained after centrifugation were washed five times with wash buffer (50 mM Tris, pH7.5, 7 mM MgCl2, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride).

Western Blotting

Antibodies used for immunoblotting including rabbit polyclonal anti-ERα (1:500-1000, abcam), mouse monoclonal anti-MR (1:500, Developmental Studies Hybridoma Bank), mouse monoclonal anti-striatin (1:500, BD bioscience), rabbit anti-eNOS (1:1000) & anti-phospho-eNOS (serine 1177, 1:500) antibody, rabbit anti-GAPDH (1:1000) and mouse anti-beta actin (1:2000), all from Cell Signaling Technology.

Supplemental Reference

1. Lu Q, Pallas DC, Surks HK, Baur WE, Mendelsohn ME,Karas RH. Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha. *Proc Natl Acad Sci U S A*. 2004;101:17126-17131.

Table S1. Metabolic parameters of female mice after 20 weeks of normal or high fat diet.

Groups	**Normal Chow (Lean)**		**High Fat Diet (Obese)**		**High Fat Diet (Obese)**		**Statistics**
Genotypes	**EC-MR-WT**	**EC-MR-KO**	**EC-MR-WT**	**EC-MR-KO**	**EC-ERα/MR-WT**	**EC-ERα/MR-KO**	
Body Weight (g)	25.45 ± 4.426 (N=26)	26.25 ± 3.370 (N=24)	37.24 ± 5.012 (N=19)	38.15 ± 4.179 (N=21)	37.45 ± 4.094 (N=13)	37.85 ± 4.950 (N=12)	*$p<0.05$ lean vs obese
Fasting Glucose (mg/dL)	163.09 ± 20.61 (N=11)	154.00 ± 3.99 (N=6)	234.44 ± 51.550 (N=15)	215.75 ± 36.656 (N=16)	230.43 ± 33.351 (N=7)	238.00 ± 41.328 (N=7)	*$p<0.05$ lean vs obese
Plasma Aldosterone (pg/mL)	147.57 ± 69.057 (N=7)	200.49 ± 60.067 (N=8)	215.360 ± 137.170 (N=10)	205.371 ± 145.906 (N=7)	325.19 ± 100.638 (N=5)	359.60 ± 159.510 (N=7)	*$p<0.05$ lean vs obese

Values presented as mean ± standard deviation. Lean animals were compared to all of the obese animals by two-factor ANOVA compared with diet and genotype as variables. There is no significant genotype effect on aldosterone levels.

Table S2. Vascular function parameters of obese female mice

Groups	High Fat Diet (Obese)			
Genotypes	**EC-MR-WT**	**EC-MR-KO**	**EC-ERα/MR-WT**	**EC-ERα/MR-KO**
Estimated lumen diameter (um)	208.15 ± 23.248 (N=15)	205.05 ± 27.275 (N=12)	212.38 ± 27.694 (N=8)	216.83 ± 19.762 (N=10)
Phenylephrine constriction (mN)	3.42 ± 0.805 (N=15)	3.95 ± 0.919 (N=12)	3.28 ± 1.273 (N=8)	3.30 ± 0.907 (N=10)
Phenylephrine constriction with LNAME (mN)	4.37 ± 1.314 (N=7)	3.88 ± 1.492 (N=7)	4.69 ± 0.606 (N=6)	3.75 ± 0.649 (N=10)

Values presented as mean ± standard deviation.

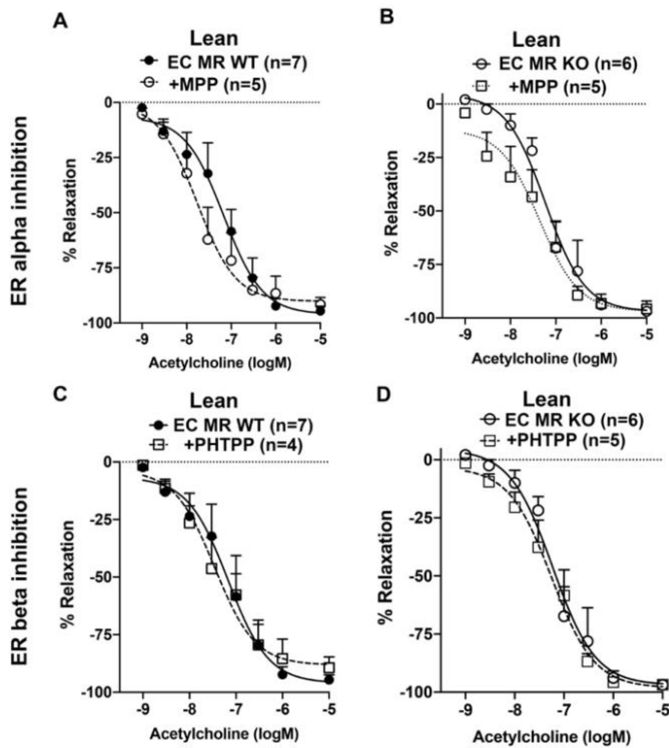

Figure S1. Estrogen receptor inhibition has no effect on endothelial-dependent vasodilation in lean female mice. Female EC-MR intact (EC-MR-WT) and EC-MR-KO mice were fed normal chow (lean) for 20 weeks and endothelial dependent vasodilation was measured *ex vivo* using acetylcholine (ACh). (**A**) Arteries were incubated with vehicle or ERα inhibitor MPP for 30 minutes prior to Ach concentration response. No difference in vasodilation in lean females comparing EC-MR-WT versus EC-MR-WT incubated with MPP. (**B**) Arteries were incubated with vehicle or ERβ inhibitor PHTPP for 30 minutes prior to Ach concentration response. No difference in vasodilation in lean females comparing EC-MR-WT versus EC-MR-WT incubated with PHTPP.

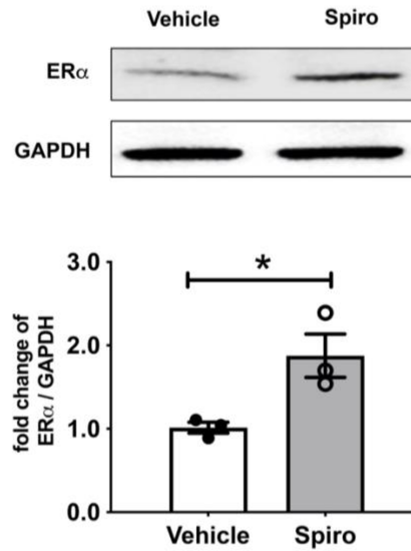

Figure S2. MR inhibition increases endogenous ERα expression in endothelial cells. Human endothelial cells that endogenously express ERα (HUVEC) were treated with vehicle or the MR inhibitor spironolactone for 20 hours and the amount of ERα protein was measured via western blot. *$p<0.05$ via unpaired student's t-test.

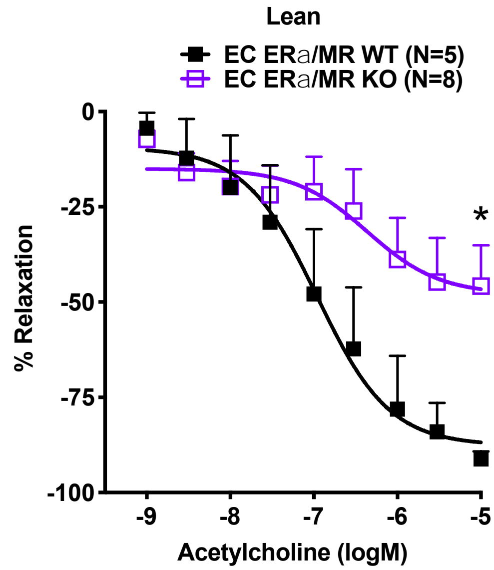

Figure S3. Combined endothelial deletion of MR and ERα significantly impairs vasodilation in lean females. Female EC- ERα/MR intact (EC-ERα/MR-WT) and EC-ERα/MR-KO mice were fed normal chow (lean) for 20 weeks and endothelial dependent vasodilation was measured *ex vivo* using acetylcholine (ACh). *$p<0.05$ via 2 way ANOVA with Bonferroni post hoc test.