Supplemental Materials

Endothelial PPARγ Protects from Angiotensin II-induced Endothelial Dysfunction in Adult Offspring Born from Pregnancies Complicated by Hypertension

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Methods

Blood pressure measurements: Systolic blood pressure (SBP) was measured using tail-cuff plethysmography (Visitech 2000 system).[1] Measurements were made in a quiet facility and at the same time each day. Briefly, mice were trained for 3 weeks before collecting baseline blood pressure for 1 week. Blood pressure was monitored 5 days a week throughout gestation in C57BL/6J mice or during Ang II infusion in adult offspring. During blood pressure measurement each day, the first 10 recordings were discarded, to allow mice to acclimatize to the warm 37^{o}C platform. After acclimatization, 30 blood pressure recordings were acquired for each mouse. At least 15 (50% successful) separate pressure readings were averaged each day for each individual mouse. For each mouse and day, the number of attempts, number of successful blood pressure readings and mean of that day's measurements were recorded.

Pregnancy studies: Baseline blood pressures were recorded via tail-cuff plethysmography as described above. Mice were randomly assigned between experimental groups based on baseline SBP, implanted with osmotic mini-pumps (Alzet 1004) to deliver AVP (24ng/kg/hr, Sigma Aldrich V9879) or saline (vehicle), and allowed to recover for 3 days before mating. E-V290M males were put in female cages for one night and blood pressure recordings by tail-cuff resumed on GD 3. On GD 15, mice were transferred to metabolic cages for 48 hours for two 24-hr urine collections.[2]

Measurement of Urinary Protein: Concentration of protein in the urine was measured using a commercially available bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, 23225) as per manufacturer's protocol.

Wire myograph studies: Wire myograph studies were conducted in conduit vessels as previously described.[1, 3] Briefly, mice were euthanized with pentobarbital (50 mg/mouse i.p.); carotid arteries were quickly removed and placed in Krebs buffer (NaCl 118.3 mmol/L, KCl 4.7 mmol/L, $CaCl_2$ 2.5 mmol/L, $MgSO_4$ 1.2 mmol/L, KH_2PO_4 1.2 mmol/L, $NaHCO_3$ 25 mmol/L, glucose 11 mmol/L). Carotid artery was cut into 3-4 mm sections in length and suspended in an organ chamber containing Krebs buffer supplied with 95% O_2/5% CO_2 and maintained at 37°C. Isometric tension on the suspended vessels was measured using a forced transducer. After equilibrating the carotid arteries to 0.25g for 45 minutes, contractile response to potassium chloride (10-100mmol/L) was assessed. The arteries were then pre-constricted with thromboxane A2 mimetic U46619. After a plateau was reached, concentration-response curves were determined in these arteries for acetycholine (ACh) (0.001–100 µmol/L) and sodium nitroprusside (SNP) (0.001–100 µmol/L). For some experiments, carotid arteries were incubated with tempol (1mmol/L), apocynin (100µmol/L), Rho-kinase inhibitor Y-27632 (1µmol/L) or ROCK-2 specific inhibitor SLX-2119 (1µmol/L) for 30 minutes before concentration response curves for ACh were assessed.

Western Blotting: Western blotting was performed on protein extracted from aorta. Protein was extracted in lysis buffer containing 50mmol/L Tris Cl, 0.1mmol/L EDTA, 1% w/v NA deoxycholic acid, 1% w/v NP-40 and 0.1% SDS, with protease (Roche) and phosphatase (Roche) inhibitors. Samples were lysed, centrifuged at 13000rpm for

15mins at 4^{o}C and supernatants were collected. The protein concentration was determined by Lowry assay (Biorad). Equal amount of tissue lysates (30µg) were separated by SDS-PAGE (8-10% gel) and transferred onto a nitrocellulose membrane. The membranes were blocked appropriately and incubated with primary antibodies at 4^{o}C overnight and visualized using horse-radish peroxidase-conjugated secondary antibody (1:10000 at RT for 1 hour). Primary antibodies against Nrf2 (ab62352), NQO1 (ab34173), β-actin (ab8227) were purchased from Abcam, and RhoA (#2117), ROCK1 (#4035), ROCK2 (#9029) and phosphorylated-MYPT1 (#5163) used in these studies were purchased from Cell Signaling.

Drugs: Acetylcholine (Cat #A6625), sodium nitroprusside (Cat #1614501), angiotensin II (Cat #A9525), potassium chloride (Cat #P9333), serotonin (Cat #14927), endothelin-1 (E7764) were purchased from Sigma Aldrich. All these reagents were dissolved in saline. U46619 (Cat #16450, Cayman Chemical) was dissolved in ethanol and subsequent dilutions were made in saline. Tempol, apocynin, Y-27632 and SLX-2119 were obtained from Sigma Aldrich (Cat #176141), EMD Millipore Calbiochem (Cat #178385), Tocris (Cat #1254) and ApexBio (Cat #A3825) respectively.

Statistical Analysis: All data are expressed as mean ± SEM. Data were analyzed using one- or two-way ANOVA (repeated measures when appropriate) using Bonferroni post hoc tests. $P \leq 0.05$ was considered statistically significant. Data were analyzed using Graphpad Prism 7 Software.

References

1. Nair AR, Agbor LN, Mukohda M, Liu X, Hu C, Wu J, Sigmund CD. Interference With Endothelial PPAR (Peroxisome Proliferator-Activated Receptor)-gamma Causes Accelerated Cerebral Vascular Dysfunction in Response to Endogenous Renin-Angiotensin System Activation. *Hypertension*. 2018;72:1227-1235
2. Santillan MK, Santillan DA, Scroggins SM, Min JY, Sandgren JA, Pearson NA, Leslie KK, Hunter SK, Zamba GK, Gibson-Corley KN, Grobe JL. Vasopressin in preeclampsia: a novel very early human pregnancy biomarker and clinically relevant mouse model. *Hypertension*. 2014;64:852-859
3. Hu C, Keen HL, Lu KT, Liu X, Wu J, Davis DR, Ibeawuchi SC, Vogel S, Quelle FW, Sigmund CD. Retinol-binding protein 7 is an endothelium-specific PPARgamma cofactor mediating an antioxidant response through adiponectin. *JCI Insight*. 2017;2:e91738

Supplemental Tables

	MALES				FEMALES			
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP
5wk	16.3±0.7	16.7±0.2	17.4±0.3	17.6±0.4	13.8±0.7	14.2±0.3	14.6±0.2	14.8±0.3
21wk	24.4±1.6	26.1±0.6	27.7±0.7	28.3±0.6	21.2±0.6	20.3±0.1	21.00±0.3	20.8±0.4

Table S1: Body Weight in Offspring
Body weights were measured in male and female offspring at 5 weeks and 21 weeks of age. All data are presented as mean±SEM.

MALES								
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL + TEMPOL	E-V290M SAL + TEMPOL	NT AVP + TEMPOL	E-V290M AVP + TEMPOL
EC_{50} (logM)	-7.36±0.06	-7.30±0.08	-7.00±0.13	-6.84±0.14	-7.39±0.08	-7.37±0.09	-7.04±0.14	-7.17±0.06
E_{max} (%)	78.79±2.3	71.05±2.9	71.34±4.4	50.44±3.7	81.47±3.1	81.99±3.3	78.20±4.8	76.62±2.4
FEMALES								
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL + TEMPOL	E-V290M SAL + TEMPOL	NT AVP + TEMPOL	E-V290M AVP + TEMPOL
EC_{50} (logM)	-7.55±0.06	-7.33±0.09	-7.33±0.07	-7.05±0.11	-7.56±0.08	-7.34±0.10	-7.24±0.10	-7.15±0.09
E_{max} (%)	83.10±2.2	84.15±3.0	71.28±2.0	66.54±3.4	87.85±3.0	90.37±3.4	85.20±3.5	77.05±3.0

Table S2: *Effect of Tempol on Vascular Function*
Dose-dependent vasorelaxation was measured to ACh in carotid arteries with or without incubation with superoxide scavenger, tempol. 4-parameter non-linear curve fitting was used to calculate EC_{50} and maximum responses for all relaxation curves. Baseline values were constrained to zero. All data are presented as mean±SEM analyzed by 3-way ANOVA.

ANOVA results Males:
EC_{50} – AVP: P<0.001; Tempol: P=0.1248; Genotype: P=0.7096; AVP X Tempol: P=0.3659; AVP X Genotype: P=0.8654, Tempol X Genotype: P=0.2722; AVP X Genotype X Tempol: P=0.4017. E_{max} – AVP: P<0.05; Tempol: P<0.001; Genotype: P<0.05; AVP X Tempol: P=0.0647; AVP X Genotype: P=0.1385, Tempol X Genotype: P<0.05; AVP X Genotype X Tempol: P=0.2751.

ANOVA results Females: EC_{50} – AVP: P<0.001; Tempol: P=0.9065; Genotype: P<0.05; AVP X Tempol: P=0.9688; AVP X Genotype: P=0.7842, Tempol X Genotype: P=0.4608; AVP X Genotype X Tempol: P=0.4608. E_{max} – AVP: P<0.001; Tempol: P<0.001; Genotype: P=0.2834; AVP X Tempol: P=0.1285; AVP X Genotype: P=0.0676, Tempol X Genotype: P=0.8202; AVP X Genotype X Tempol: P=0.5692.

MALES								
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL + APO	E-V290M SAL + APO	NT AVP + APO	E-V290M AVP + APO
EC_{50} (logM)	-7.14±0.10	-7.06±0.11	-7.05±0.10	-6.88±0.08	-7.38±0.07	-7.05±0.10	-7.27±0.15	-7.24±0.07
E_{max} (%)	77.94±3.9	67.28±3.7	70.59±3.2	68.11±3.0	87.45±2.9	73.02±3.4	78.80±4.8	84.89±2.6
FEMALES								
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL + APO	E-V290M SAL + APO	NT AVP + APO	E-V290M AVP + APO
EC_{50} (logM)	-7.44±0.06	-7.23±0.09	-7.24±0.08	-6.85±0.10	-7.35±0.06	-7.39±0.07	-7.34±0.10	-7.14±0.04
E_{max} (%)	90.44±2.5	81.09±3.9	78.86±2.8	71.00±3.4	83.47±2.3	86.97±2.6	84.84±3.5	82.03±1.7

Table S3: *Effect of Apocynin on Vascular Function*
Dose-dependent vasorelaxation was measured to ACh in carotid arteries with or without incubation with apocynin. 4-parameter non-linear curve fitting was used to calculate EC_{50} and maximum responses for all relaxation curves. Baseline values were constrained to zero. All data are presented as mean±SEM analyzed by 3-way ANOVA.

ANOVA Results Male: EC_{50} – AVP: P=0.5134; Apocynin: P<0.05; Genotype: P<0.05; AVP X Apocynin: P=0.236; AVP X Genotype: P=0.4707, Apocynin X Genotype: P=0.7039; AVP X Genotype X Apocynin: P=0.189. E_{max} – AVP: P=0.7423; Apocynin: P<0.001; Genotype: P<0.05; AVP X Apocynin: P=0.3381; AVP X Genotype: P<0.05, Apocynin X Genotype: P=0.6331; AVP X Genotype X Apocynin: P=0.2289.

ANOVA Results Females: EC_{50} – AVP: P<0.05; Apocynin: P=0.0524; Genotype: P<0.05; AVP X Apocynin: P=0.1643; AVP X Genotype: P=0.0738, Apocynin X Genotype: P=0.0623; AVP X Genotype X Apocynin: P=0.7881. E_{max} – AVP: P<0.05; Apocynin: P=0.0647; Genotype: P=0.0562; AVP X Apocynin: P<0.05; AVP X Genotype: P=0.5565, Apocynin X Genotype: P<0.05; AVP X Genotype X Apocynin: P=0.3455.

MALES								
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL + Y-27632	E-V290M SAL + Y-27632	NT AVP + Y-27632	E-V290M AVP + Y-27632
EC_{50} (logM)	-7.28±0.04	-7.35±0.05	-7.34±0.08	-6.71±0.16	-7.37±0.04	-7.42±0.07	-7.36±0.08	-7.17±0.08
E_{max} (%)	89.28±1.6	86.08±2.1	80.88±2.9	58.10±5.3	91.09±1.7	90.82±3.1	87.52±3.0	83.67±3.3
FEMALES								
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL + Y-27632	E-V290M SAL + Y-27632	NT AVP + Y-27632	E-V290M AVP + Y-27632
EC_{50} (logM)	-7.21±0.08	-7.16±0.09	-7.25±0.05	-7.08±0.10	-7.46±0.07	-7.37±0.08	-7.51±0.11	-7.41±0.06
E_{max} (%)	80.91±3.3	77.03±3.7	81.69±2.0	65.15±3.3	95.96±3.2	88.73±3.5	81.89±4.0	95.31±2.4

Table S4: *Effect of Rho-kinase Inhibition on Vascular Function*
Dose-dependent vasorelaxation was measured to ACh in carotid arteries with or without incubation with Y-27632. 4-parameter non-linear curve fitting was used to calculate EC_{50} and maximum responses for all relaxation curves. Baseline values were constrained to zero. All data are presented as mean±SEM analyzed by 3-way ANOVA.

ANOVA Results Males: EC_{50} – AVP: P<0.01; Y-27632: P<0.05; Genotype: P<0.01; AVP X Y-27632: P=0.1928; AVP X Genotype: P<0.01, Y-27632 X Genotype: P=0.0933; AVP X Genotype X Y-27632: P=0.0684. E_{max} – AVP: P<0.001; Y-27632: P<0.001; Genotype: P<0.01; AVP X Y-27632: P<0.01; AVP X Genotype: P<0.05, Y-27632 X Genotype: P<0.05; AVP X Genotype X Y-27632: P=0.0854.

ANOVA Results Females: EC_{50} – AVP: P=0.8324; Y-27632: P<0.001; Genotype: P=0.0968; AVP X Y-27632: P=0.5836; AVP X Genotype: P=0.5836, Y-27632 X Genotype: P=0.8989; AVP X Genotype X Y-27632: P=0.6423. E_{max} – AVP: P=0.0591; Y-27632: P<0.0001; Genotype: P=0.1394; AVP X Y-27632: P=0.6984; AVP X Genotype: P=0.3954, Y-27632 X Genotype: P<0.05; AVP X Genotype X Y-27632: P<0.001.

MALES								
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL + SLX2119	E-V290M SAL + SLX2119	NT AVP + SLX2119	E-V290M AVP + SLX2119
EC_{50} (logM)	-7.25±0.05	-7.38±0.05	-7.43±0.08	-6.76±0.12	-7.77±0.11	-7.75±0.11	-7.70±0.13	-7.75±0.14
E_{max} (%)	86.25±2.0	85.00±2.0	84.29±2.8	70.87±4.4	96.98±3.9	89.76±3.9	89.92±4.2	90.81±4.8
FEMALES								
	NT SAL	E-V290M SAL	NT AVP	E-V290M AVP	NT SAL + SLX2119	E-V290M SAL + SLX2119	NT AVP + SLX2119	E-V290M AVP + SLX2119
EC_{50} (logM)	-7.15±0.09	-7.25±0.07	-7.26±0.05	-7.20±0.10	-7.32±0.10	-7.73±0.10	-7.75±0.20	-7.41±0.08
E_{max} (%)	78.61±3.1	82.51±2.8	80.96±2.0	64.74±3.4	92.16±4.0	96.18±3.6	85.19±6.3	92.71±3.1

Table S5: *Effect of ROCK2 Inhibition on Vascular Function*
Dose-dependent vasorelaxation was measured to ACh in carotid arteries with or without incubation with SLX-2119. 4-parameter non-linear curve fitting was used to calculate EC_{50} and maximum responses for all relaxation curves. Baseline values were constrained to zero. All data are presented as mean±SEM analyzed by 3-way ANOVA.

ANOVA Results Males: EC_{50} – AVP: P=0.1021; SLX-2119: P<0.001; Genotype: P=0.1021; AVP X SLX-2119: P=0.2264; AVP X Genotype: P<0.05, SLX-2119 X Genotype: P=0.0705; AVP X Genotype X SLX-2119: P<0.01. E_{max} – AVP: P<0.05; SLX-2119: P<0.01; Genotype: P=0.0574; AVP X SLX-2119: P=3404; AVP X Genotype: P=0.6975, SLX-2119 X Genotype: P=0.4281; AVP X Genotype X SLX-2119: P=0.0655.

ANOVA Results Females: EC_{50} – AVP: P=0.5827; SLX-2119: P<0.001; Genotype: P=0.7215; AVP X SLX-2119: P=0.8711; AVP X Genotype: P<0.01, SLX-2119 X Genotype: P=0.9224; AVP X Genotype X SLX-2119: P=0.0694. E_{max} – AVP: P<0.05; SLX-2119: P<0.0001; Genotype: P=0.9427; AVP X SLX-2119: P=0.6476; AVP X Genotype: P=0.1395, SLX-2119 X Genotype: P<0.05; AVP X Genotype X SLX-2119: P<0.05.

Supplemental Figures

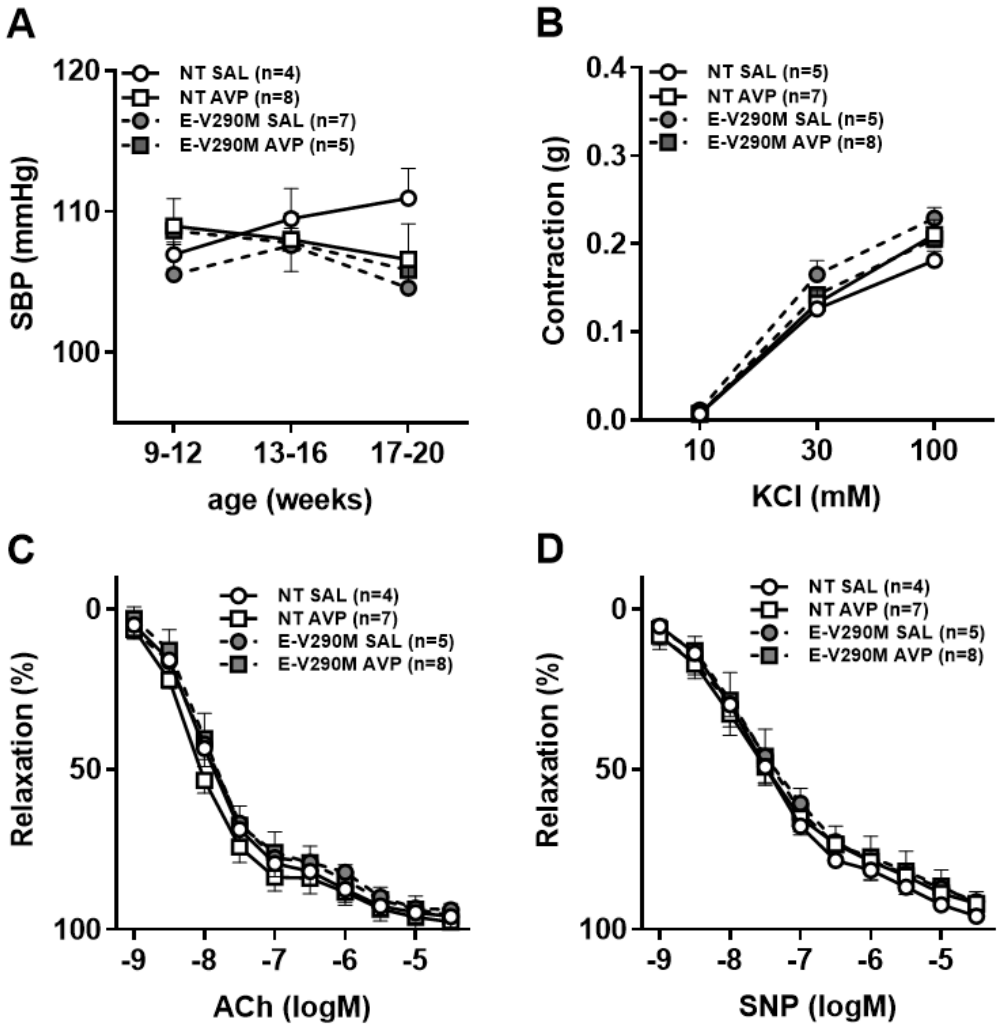

Figure S1: *Blood Pressure and Vascular Reactivity in Adult Offspring*
A) Systolic blood pressure measured using tail-cuff plethysmography in adult NT and E-V290M offspring born from saline or AVP-infused pregnancies (NT Sal, n=4; NT AVP, n=8; E-V290M Sal, n=7; E-V290M AVP, n=5), B) Dose-dependent vasoconstrictive responses to KCl in carotid arteries from adult NT and E-V290M offspring born from saline or AVP-infused pregnancies (NT Sal, n=5; NT AVP, n=7; E-V290M Sal, n=5; E-V290M AVP, n=8). Cumulative concentration-dependent relaxation responses to B) ACh and D) SNP in carotid arteries from Ang II-treated adult male NT and E-V290M offspring born from saline or AVP-infused pregnancies (NT Sal, n=4; NT AVP, n=7; E-V290M Sal, n=5; E-V290M AVP, n=8). All data are presented as mean±SEM analyzed by two-way ANOVA. P>0.05.

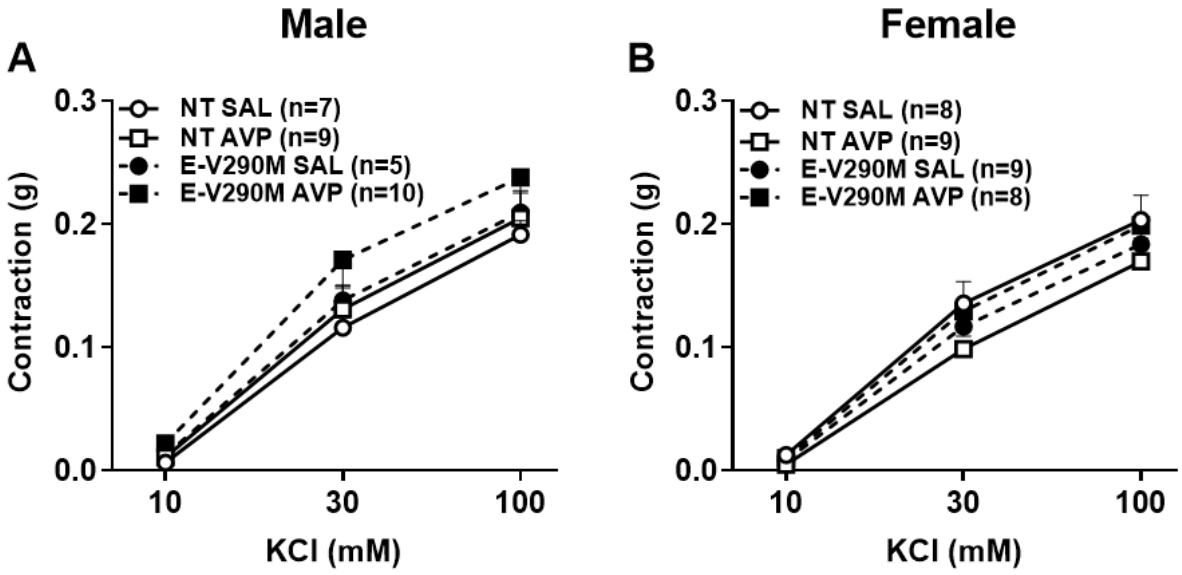

Figure S2: *Angiotensin II Sensitivity in Adult Offspring.*
Dose-dependent vasoconstrictive responses to KCl in carotid arteries from A) adult male NT and E-V290M offspring born from saline or AVP-infused pregnancies (NT Sal, n=7; NT AVP, n=9; E-V290M Sal, n=5; E-V290M AVP, n=10) and B) adult females (NT Sal, n=8; NT AVP, n=9; E-V290M Sal, n=9; E-V290M AVP, n=8). All data are presented as mean±SEM analyzed by two-way ANOVA. P>0.05.

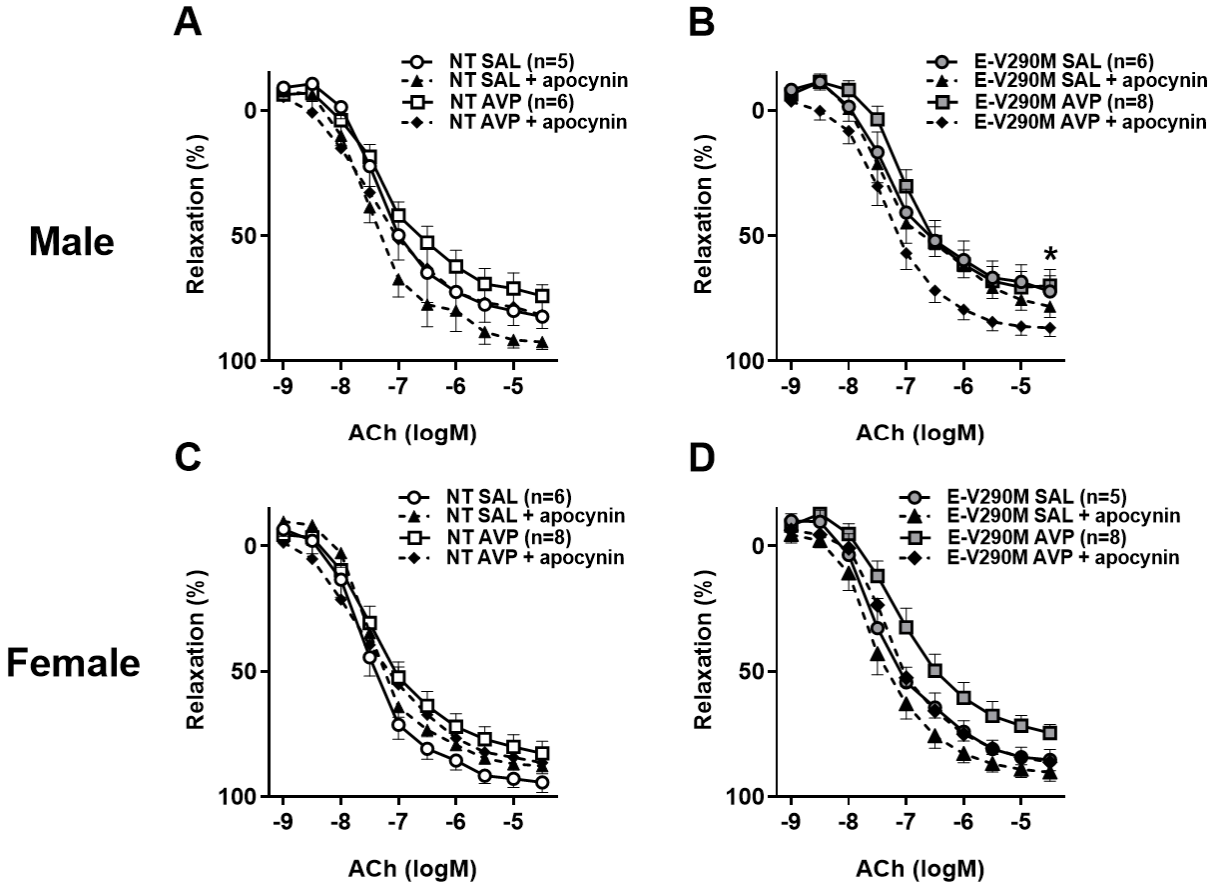

Figure S3: *Endothelial Function in Adult Offspring – Role of ROS: Apocynin.*
Cumulative concentration response curves for ACh in carotid arteries from adult males with and without apocynin (100 μmol/L, 30mins), for A) NT Sal (n=5) and NT AVP (n=6), and B) E-V290M Sal (n=6) and E-V290M AVP (n=8). Cumulative concentration response curves for ACh in carotid arteries from adult females with and without apocynin for C) NT Sal (n=6) and NT AVP (n=8), and D) E-V290M Sal (n=5) and E-V290M AVP (n=8). Data are presented as mean±SEM analyzed by two-way ANOVA with repeated measures. *P<0.05 vs. E-V290M AVP + apocynin.