Materials and Methods:

Mice: All animals were handled in accordance with NIH standards, and the procedures were approved by the Tufts Medical Center Institutional Animal Care and Use Committee. C57BL/6 mice (WT) were purchased from The Jackson Laboratory and SMC-MR-deficient mice were generated by crossing mice with critical exons of the MR gene flanked by loxP sites ($MR^{f/f}$)[1] with SMA-Cre-ERT2 mice (smooth muscle actin promoter driving expression of the Cre-ERT2 recombinase that is activated by tamoxifen[2]), as previously described[1]. Male $MR^{f/f}$/SMA-Cre-ER^{T2}+ (SMC-MR-KO) and $MR^{f/f}$/SMA-Cre-ER^{T2}– littermates (MR-Intact) were induced by intraperitoneal injection of 1 mg Tamoxifen daily for 5 days at age 6–8 weeks and studies were performed 4 weeks post-induction to allow for MR excision and degradation as previously confirmed[1].

Wire carotid injury model and Evan's blue staining: The wire injury protocol has been previously described in detail[3, 4]. One day prior to carotid injury, vehicle or aldosterone infusion pumps (Sigma-Aldrich, 240µg/kg/d, dissolved in 100% ethanol and diluted in saline) or vehicle (11% ethanol in saline). Left carotid endothelial denudation vascular injury was then produced and a Bromodeoxyuridine (BrDU, Sigma-Aldrich, 25 mg/kg/d) infusion pump was placed subcutaneously. The right carotid artery served as an uninjured control in each mouse. Two weeks after injury, both the injured and uninjured control carotid arteries were perfusion-fixed and BrDU positive cells, medial area and extracellular matrix quantified histologically as described[3, 4]. All measurements were made by treatment- and genotype-blinded investigators on intact sections in which complete endothelial denudation could be first confirmed by BRdU staining of all luminal EC. Ten to 16 animals were included in each group. Re-endothelialization was assessed by staining with Evans Blue dye (Sigma) as described[5]. Immediately after injury and at specific times after, 50 µL of 5% Evans blue diluted in saline was injected into the tail vein 10 minutes before euthanasia, followed by perfusion-fixation. The carotid artery was then dissected, cut longitudinally, and photographed under a dissecting microscope. The percent of the total carotid vessel area that is stained in blue was calculated using images acquired by a treatment blinded investigator using ImagePro 6.2 software.

In vivo VEGF-blocking antibody injections: Mice were injected intraperitoneally with VEGFR1-blocking antibody (clone MF1, Imclone Systems, NY) or VEGFR2-blocking antibody (clone DC 101, ATCC, VA) as described elsewhere[6-8] at 35 mg/kg diluted in phosphate buffered saline (PBS). Injection was performed at the time of injury and every 2 days for a total of 5 injections. One group of control mice was treated with PBS alone and another group with control IgG antibody (Innovative Research, #Ir-RT-GF, 35 mg/kg in PBS). As there was no detectable difference in any baseline or post-infusion characteristics (blood pressure, body weight, serum aldosterone levels, or vascular

injury responses) between the PBS and the PBS/IgG treated mice, the data from these 2 control groups was pooled and referred to as “control IgG”.

Immunohistochemistry: Serial sections of carotid arteries 14 days after wire injury of WT mice or SMC-MR-KO and MR Intact mice treated with vehicle or aldosterone were stained with anti-VEGFR1 rabbit polyclonal IgG (Santa Cruz, cat # sc-9029, 1:100) according to the Santa Cruz protocol for paraffin embedded tissues for this antibody. Medial VEGFR1 positive area was quantified using computerized morphometric analysis by a blinded investigator as for the fibrosis quantification described previously[3]. The media was identified by comparing parallel carotid sections stained with endothelial marker CD31 (Abcam, cat # 28365 rabbit polyclonal, 1:100) and SMC marker smooth muscle–specific α-actin (Sigma-Aldrich, monoclonal, cat # F3777, 1:100). Six to 8 mice were included in each group.

Carotid SMC assays: Primary mouse carotid SMC from passages 4 to 12 were treated with aldosterone, eplerenone, and/or antibody (control antibody (Innovative research, Sprague Dawley Rat IgG), VEGFR1antibody (Clone MF1, Imclone systems), or VEGFR2 antibody (Clone DC101, ATCC)) at 4.5 micrograms per 100 microliter well. After 48 hours cell number was quantified using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instructions. For all *in vitro* experiments, aldosterone and eplerenone were dissolved in DMSO and appropriate vehicle controls were used. Each treatment was performed in triplicate in each experiment, averaged, and the data expressed as the percent increase versus control vehicle treated cells. Each experiment was performed at least 5 times.

Blood pressure measurement: BP was previously extensively characterized in SMC-MR-KO mice and MR Intact controls by telemetry revealing no difference in basal BP or BP under a variety of aldosterone or salt conditions at the age used for the carotid injury studies (3-4 months)[1]. Since telemetry devices are inserted via the carotid artery, they cannot be used in conjunction with the carotid wire injury model. BP was also measured in the specific mice undergoing the injury procedure by tail cuff plethysmography prior to carotid injury and again after 1 week of vehicle or aldosterone infusion using the Kent coda system by a well validated protocol that correlates closely with telemetry results as described in detail[3]. Briefly, mice were trained twice daily with 20 tail cuff inflations for at least 3 days. The morning after training, BP was measured with 20 cuff inflations, the values averaged, rare outliers (±2 SD from mean) eliminated, and systolic BP recorded as the mean of the remaining measurements (provided there were at least 5 remaining values). BP data analysis was performed after the termination of the experiment by a genotype- and treatment-blinded investigator.

Quantitative Real Time PCR: RNA was isolated from mouse aortas treated ex vivo or from carotid SMC treated *in vitro* with vehicle or aldosterone for 8 hours as described[3]. RNA was reverse transcribed and PlGF and/or VEGFR1 mRNA was quantified by real

time PCR with gene-specific primers[3]. Each treatment was performed in at least duplicate and averaged for each experiment. Each experiment was performed at least 3 times.

PlGF Protein Assay: Media was collected from mouse aortas after treatment with vehicle or aldosterone for 8 hours and PlGF levels were measured by Quantikine mouse PlGF2 ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. N=3 vessels per treatment.

Serum Aldosterone Assay: Mouse serum aldosterone levels were measured by COAT-A-COUNT Aldosterone Radioimmunoassay (Siemans Medical Solutions Diagnostics, Los Angeles,CA) according to the manufacturer's instructions.

Statistics: Values are reported as mean ± SEM. Within-group differences were assessed with 1-way ANOVA with Student-Newman-Keuls post-hoc test or 1-way ANOVA on ranks with Dunn's method post-hoc test when appropriate. Carotid injury analyses were performed by 2-way ANOVA with Student-Newman-Keuls post-hoc test. $P < 0.05$ was considered significant.

Reference List

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