Aldosterone Promotes Vascular Remodeling by Direct Effects on Smooth Muscle Cell Mineralocorticoid Receptors
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Vascular remodeling occurs in response to endothelial injury, resulting in smooth muscle cell (SMC) proliferation and vascular fibrosis. We previously demonstrated that the blood pressure–regulating hormone aldosterone enhances vascular remodeling in mice at sites of endothelial injury in a placental growth factor–dependent manner. We now test the hypothesis that SMC mineralocorticoid receptors (MRs) directly mediate the remodeling effects of aldosterone and further explore the mechanism.

**Approach and Results**—A wire-induced carotid injury model was performed in wild-type mice and mice with inducible SMC-specific deletion of the MR. Aldosterone did not affect re-endothelialization after injury in wild-type mice. Deletion of SMC-MR prevented the 79% increase in SMC proliferation induced by aldosterone after injury in MR-Intact littermates. Moreover, both injury-induced and aldosterone-enhanced vascular fibrosis were attenuated in SMC-specific MR knockout mice. Further exploration of the mechanism revealed that aldosterone-induced vascular remodeling is prevented by in vivo blockade of the placental growth factor–specific receptor, type 1 vascular endothelial growth factor receptor (VEGFR1), the receptor for placental growth factor. Immunohistochemistry of carotid vessels shows that the induction of VEGFR1 expression in SMC after vascular injury is attenuated by 72% in SMC-specific MR knockout mice. Moreover, aldosterone induction of vascular placental growth factor mRNA expression and protein release are also prevented in vessels lacking SMC-MR.

**Conclusions**—These studies reveal that SMC-MR is necessary for aldosterone-induced vascular remodeling independent of renal effects on blood pressure. SMC-MR contributes to induction of SMC VEGFR1 in the area of vascular injury and to aldosterone-enhanced vascular placental growth factor expression and hence the detrimental effects of aldosterone are prevented by VEGFR1 blockade. This study supports exploring MR antagonists and VEGFR1 blockade to prevent pathological vascular remodeling induced by aldosterone. *(Arterioscler Thromb Vasc Biol. 2014;34:355-364.)*

**Key Words:** aldosterone ◼ myocytes, smooth muscle ◼ receptors, mineralocorticoid ◼ placental growth factor ◼ vascular endothelial growth factor receptor-1
membrane VEGF type 1 and type 2 receptors (VEGFR1 and VEGFR2) modulate vascular SMC and EC function via the transmembrane family of secreted proteins that contribute to angiogenesis. These novel therapeutic strategies could provide new insights into the potential molecular downstream mechanisms that regulate vascular remodeling in vivo.

Results

Aldosterone Does Not Alter the Rate of Re-Endothelialization After Vascular Injury

We set out to explore the mechanism by which aldosterone infusion enhances vascular remodeling specifically at sites of vascular injury without significantly changing BP. It has been suggested that the rate of endothelial regrowth after arterial injury determines the degree of vascular remodeling with accelerated re-endothelialization leading to an attenuated injury response. Thus, we first examined the effect of aldosterone on the rate of re-endothelialization in a mouse carotid wire injury model. In this model, an aldosterone or vehicle infusion pump is inserted 1 day before carotid endothelial denudation by wire injury (Figure 1A). After wire-induced carotid injury, Evans blue dye is infused to mark the areas of denuded carotid endothelium. Representative images of the residual area reveal no significant difference in the percentage of area covered with endothelium in arteries from aldosterone compared with vehicle-treated mice at all time points after injury (Figure 1B). These results suggest that aldosterone is not enhancing the vascular remodeling response by altering EC proliferation or migration and may instead be acting on MR elsewhere in the vessel so we next focused on the SMCs.

Aldosterone Enhances Vascular Injury by Direct, BP-Independent Effects on SMC-MR

The role of SMC-MR in aldosterone-stimulated vascular injury was examined directly using a mouse model with MR genetically deleted in adulthood specifically from SMC (SMC-MR-KO) compared with MR-intact littermate controls. Prior studies reveal that at 3 months, SMC-MR-KO mice have no significant difference in systemic BP with or without aldosterone infusion when compared with MR-intact controls as measured by telemetry. This is confirmed by tail-cuff plethysmography in the specific mice used for carotid injury that cannot have concurrent telemetry (Table). Mice underwent the carotid injury protocol (Figure 1A) with insertion of a bromodeoxyuridine infusion pump at the time of injury to mark proliferating cells, and vascular remodeling was quantified 14 days after injury. Aldosterone was infused at a low dose that increases circulating aldosterone levels significantly and similarly in both genotypes to levels consistent with those seen in patients with cardiovascular disease. This had no effect on systolic BP or body weight in the mice (Table). In uninjured vessels, there is minimal SMC proliferation, as measured by medial bromodeoxyuridine positive nuclei, regardless of the presence of SMC-MR or exogenous aldosterone consistent with the lack of effect of aldosterone on remodeling in the absence of endothelial damage. Vascular injury enhances SMC proliferation, even in the absence of SMC-MR (P<0.001 for injured versus uninjured), thus all further comparisons are focused on the SMCs.

Materials and Methods

Materials and Methods are available in the online-only Supplement.
made between the injured vessels only. In MR-intact mice, aldosterone significantly enhances SMC proliferation after injury (Figure 2A), as we previously published in wild-type C57Bl/6 mice. However, aldosterone fails to promote SMC proliferation in SMC-MR-KO mice (Figure 2A). Aldosterone infusion also significantly enhances injury-induced vascular fibrosis in MR-intact mice but not in SMC-MR-KO mice (Figure 2B). Interestingly, even in the absence of excess aldosterone, SMC-MR deficiency attenuates vascular fibrosis, supporting the concept that SMC-MR contributes to the fibrotic response to vascular injury in the presence of physiological and pathological levels of aldosterone (Figure 2B). Finally, vascular injury causes an increase in vessel medial area that is enhanced in the presence of excess aldosterone in MR-intact but not in SMC-MR-KO mice (Figure 2C). Taken together, these data support the new concept that aldosterone enhances vascular remodeling by direct effects on MR in SMCs and that SMC-MR contributes to basal and aldosterone-enhanced vascular fibrosis in this injury model.

**VEGFR1 Blockade Prevents Aldosterone-Enhanced Vascular Remodeling In Vivo**

We previously demonstrated that aldosterone-induced vascular remodeling is dependent on the presence of the growth factor PIGF that binds specifically to VEGFR1. To explore the role of VEGF receptors in aldosterone-induced

### Table. Increased Serum Aldosterone to Pathological Levels in MR Intact and SMC-MR-KO Mice With No Change in Blood Pressure or Animal Weight

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<th>Treatment</th>
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<th>Pretreatment SBP, mm Hg</th>
<th>Pretreatment DBP, mm Hg</th>
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DBP indicates diastolic blood pressure; SBP, systolic blood pressure; and SMC-MR-KO, smooth muscle cell–specific-mineralocorticoid receptor-knockout mouse. *P<0.001 vs vehicle.
remodeling in vivo, the aldosterone-enhanced wire carotid injury model was repeated with injection of control IgG, VEGFR1- or VEGFR2-blocking antibodies. BP measurements reveal no significant effect of VEGF-blocking antibodies on BP at these doses (Table I in the online-only Data Supplement). As expected, aldosterone infusion significantly enhances injury-induced SMC proliferation, vascular fibrosis, and medial thickening in mice treated with control IgG (Figure 3). VEGFR1-blocking antibody, but not VEGFR2-blocking antibody, prevents aldosterone-enhanced SMC proliferation (Figure 3A), vascular fibrosis (Figure 3B), and medial thickening (Figure 3C). Taken together, these data suggest that VEGFR1 plays a significant and specific role in aldosterone-enhanced vascular remodeling in vivo.

Because the carotid injury studies in Figures 2 and 3 implicate direct effects of aldosterone on SMC-MR in promoting cell proliferation, we attempted to explore the mechanism using an in vitro model of mouse carotid SMC proliferation. Aldosterone enhances proliferation of primary mouse carotid SMCs in a dose-dependent manner (Figure 1A in the online-only Data Supplement) as it does for other cultured SMCs.24 However, at physiologically (1 nmol/L) and pathologically (5–10 nmol/L) relevant aldosterone concentrations, the increase in proliferation in vitro is modest (<20%) and much less than the enhanced proliferative response to the same concentration of aldosterone in vivo (70%–80%). Further studies revealed that aldosterone treatment of cultured primary mouse carotid SMCs results in a nonsignificant trend toward increased PlGF expression (much less than the 300% increase in whole vessels9) and only a modest but significant increase in VEGFR1 expression that is prevented by cotreatment with the MR-specific antagonist eplerenone (Figure 1B in the online-only Data Supplement). In vitro, VEGFR1 blockade inhibits the modest aldosterone-enhanced SMC proliferation, whereas VEGFR2 blockade prevents a significant aldosterone-induced increase in proliferation but is not significantly decreased when compared with aldosterone with IgG (Figure 1C in the online-only Data Supplement). We conclude that although the PlGF/VEGFR1 pathway is modestly activated by aldosterone and contributes
to carotid SMC proliferation in vitro, this in vitro system does not completely recapitulate the effect of aldosterone on the proliferative response to injury in vivo. Therefore, further exploration of the mechanism was performed in vivo and in whole vessels.

**SMC-MR Contributes to VEGFR1 Induction on SMCs After Vascular Injury**

In healthy vessels, VEGFR1 is expressed exclusively in the endothelium; however, upon vascular injury, VEGFR1 expression is activated on vascular SMCs by unclear mechanisms.25 To examine the vascular compartments in which VEGFR1 is expressed in the wire injury model, immunohistochemistry with VEGFR1-specific antibody was performed on uninjured and injured carotid arteries 14 days after unilateral endothelial injury. In this model, we confirm that uninjured vessels express VEGFR1 only in the endothelium, whereas VEGFR1 is expressed robustly on ECs and SMCs in injured vessels even without exogenous aldosterone administration (Figure 4A). Serial sections incubated with CD31 (an EC marker) and SMC-α actin (a SMC marker) are included in Figure 4A to confirm the identity of each cell type in the vessel.

To explore whether SMC-MR is involved in the mechanism of the injury-induced SMC expression of VEGFR1, medial VEGFR1 immunoreactivity was quantified in serial sections of the injured vessels from the study in Figure 2 (Figure 4B). VEGFR1 expression is detected in the media in all the injured vessels; however, in vessels from SMC-MR-KO, there is a significant and substantial reduction in the percentage of the vessel staining positive for VEGFR1 regardless of the presence of excess aldosterone. Taken together, these data support that SMC-MR contributes substantially to the induction of VEGFR1 expression on SMCs after a vessel is injured.

**SMC-MR Is Necessary for Aldosterone Induction of Vascular PlGF**

We previously demonstrated that aldosterone stimulates vascular PlGF transcription and release from intact and injured mouse vessels and from diseased human vessels.9 To investigate whether PlGF production is dependent on SMC-MR, whole vessels from MR-intact and SMC-MR-KO mice were treated with aldosterone ex vivo and PlGF levels were assessed. In MR-intact vessels, aldosterone increases vascular PlGF mRNA and extravascular PlGF protein >3-fold (Figure 5) as previously demonstrated in wild-type vessels.9 In vessels lacking SMC-MR, the aldosterone-induced increase in PlGF message and protein secretion are prevented, demonstrating that aldosterone regulation of vascular PlGF requires SMC-MR.
Thus, deletion of SMC-MR attenuates the local upregulation of VEGFR1 at the site of injury (Figure 4B), prevents aldosterone-induced vascular PlGF production (Figure 5), and prevents aldosterone-enhanced vascular remodeling after injury (Figure 2).

**Discussion**

In summary, we have demonstrated that SMC-MR is required for aldosterone-enhanced vascular remodeling in vivo. Aldosterone induces vascular SMC proliferation and fibrosis after injury without affecting endothelial regrowth. These adverse effects of the hormone are completely lost in mice with the MR specifically deleted from SMCs in adulthood. SMC-MR also contributes to injury-associated vascular fibrosis even without addition of exogenous aldosterone. Blockade of VEGFR1 signaling with receptor-specific antibodies also prevents aldosterone-induced SMC proliferation and vascular fibrosis after injury in vivo. Further mechanistic studies reveal that SMC-MR directly contributes to the induction of VEGFR1 expression on SMCs at the site of vascular injury and is necessary for aldosterone induction of the VEGFR1 ligand, PlGF.

Putting these data together with previously published work provides a new model by which SMC-MR contributes directly to vascular remodeling (Figure 6). This model addresses the longstanding conundrum as to why aldosterone alone has no adverse vascular phenotype unless combined with a cause for endothelial dysfunction, including high salt intake, congestive heart failure, hyperlipidemia, or localized vascular injury. In healthy vessels with an intact endothelium, VEGFR1 is expressed only on ECs and aldosterone activation of SMC-MR only modestly
increases PlGF transcription and release with no effect on SMC proliferation and fibrosis as PlGF receptors are not expressed on SMCs under these conditions (Figure 6, left). Thus in healthy vessels, aldosterone does not disrupt the quiescent SMC phenotype and vessels do not undergo remodeling even when aldosterone levels are high (eg, in healthy individuals or mice) on a low sodium diet. However, when vessels are injured or diseased, SMC-MR contributes to substantial upregulation of PlGF and to the expression of VEGFR1 on SMCs resulting in SMC proliferation and vascular fibrosis. Conversely, PlGF deficiency or VEGFR1 blockade prevents aldosterone-enhanced remodeling after injury in mice. These data from mouse models are consistent with human data demonstrating that SMC-MR–regulated pathways could be targeted to prevent adverse remodeling even when aldosterone levels are high. The use of an inducible model of MR deletion also prevents developmental effects of MR deletion from contributing to altered vascular remodeling responses. Taken together, these results support a new paradigm in which direct MR activation in the SMCs of the vasculature is responsible for enhanced vascular remodeling independent of renal MR and BP alterations.

There are several limitations and future directions to this study that should be noted. First, the wire carotid injury model in C57Bl/6 mice is a reproducible model to examine medial SMC proliferation and collagen deposition in the vessel wall. These processes are paramount in vascular remodeling induced by hypertension and aging and also occur in the setting of atherosclerosis and vascular injury in which proliferation is accompanied by SMC migration and neointima formation. Aldosterone contributes to neointimal formation in other models, but this occurs only rarely in the wire injury model in C57Bl/6 mice (<10% of mice). Thus, the direct role of SMC-MR, PlGF, and VEGFR1 in neointima formation warrants further exploration in vascular injury models with more reproducible neointimal responses. This would have important clinical implications because the aldosterone-enhanced mechanism of vascular remodeling identified here seems to dissociate SMC proliferation from endothelial regrowth, a desired situation for drugs to prevent restenosis of vascular stents in which neointima formation is an important component of the pathology. PlGF and VEGF are also vasodilators and as a result, novel cancer therapeutics that block VEGF receptors cause hypertension and further exploration of the role of VEGF signaling in vascular remodeling could have important implications for the growing patient populations treated with anti-VEGF therapy for malignancy and other conditions. In this study, we confirm that there is no substantial difference in tail-cuff BP at the concentrations of blocking antibodies or aldosterone used.

For more than half a century, aldosterone and MR have been known to regulate BP by renal sodium retention. Based on this knowledge, the detrimental cardiovascular effects of aldosterone have been attributed to secondary vascular responses to elevated BP. In this study, the low-dose aldosterone infusion enhanced vascular remodeling without increasing BP. Moreover, aldosterone-enhanced vascular remodeling is completely prevented by the specific deletion of MR from SMCs. We previously demonstrated that SMC-MR-KO mice have intact renal MR function, normal renal sodium handling, and no difference in telemetric BP at the age used for this study, which we confirm by tail-cuff BP here. The use of an inducible model of MR deletion also prevents developmental effects of MR deletion from contributing to altered vascular remodeling responses. Taken together, these results support a new paradigm in which direct MR activation in the SMCs of the vasculature is responsible for enhanced vascular remodeling independent of renal MR and BP alterations.

Figure 5. Smooth muscle cell-mineralocorticoid receptor (SMC-MR) is necessary for aldosterone regulation of vascular placental growth factor (PIGF) expression and release. A and B. Mouse aortas from 3-month-old MR-intact and SMC-MR-knockout (KO) mice were treated for 8 hours ex vivo with vehicle (gray bars) or 100 nmol/L aldosterone (black bars). A, PIGF mRNA expression was quantified by quantitative reverse transcriptase-polymerase chain reaction of vessel RNA and (B) PIGF protein secretion was quantified by ELISA of vessel conditioned media. *P<0.05 vs vehicle.
Telemetric monitoring is the gold standard for BP measurements in rodents; however, because the catheter is inserted via the carotid artery, this cannot be performed concurrently with carotid injury. We previously demonstrated by telemetry that there is no BP difference in young SMC-MR-KO mice compared with MR-intact controls at baseline or with aldosterone infusion. Here, we confirm by tail-cuff plethysmography that the substantial changes in arterial remodeling responses are not because of large changes in BP although small BP differences (≤5–10 mm Hg) cannot be accurately distinguished by this technique. Finally, understanding how the downstream signaling of MR and VEGFR1 converge in SMCs to coordinate the proliferative response to injury is an important area for future investigation. Indeed, both MR and VEGFR1 signaling have been shown to promote SMC proliferation by activation of mitogen activated protein-kinase signaling in vitro. Because the SMC-MR/PlGF/VEGFR1 mechanism identified here is specifically activated in the setting of vascular injury that is not easily reproduced in vitro, future in vivo studies will be important to explore the downstream signaling events that mediate aldosterone-induced vascular remodeling.

In conclusion, these data support a novel mechanism of aldosterone-enhanced remodeling in which direct activation of SMC-MR promotes adverse vascular remodeling by regulation of VEGFR1 and PlGF in SMCs in areas of vascular injury. This mechanism is independent of alterations in endothelial regrowth, renal MR activation, and systemic BP. Clinically, elevated serum aldosterone is increasingly common because of an association with resistant hypertension, heart failure, and obesity and aldosterone levels correlate with increased risk of myocardial infarction, stroke, and death. Furthermore, SMC-MR activation contributes to vascular fibrosis in this model even without added aldosterone and hence might contribute to vascular remodeling even in patients with normal serum aldosterone levels. Interestingly, the basal level of vascular fibrosis after injury is not significantly altered by VEGFR1 blockade, supporting an additional mechanism by which SMC-MR contributes to fibrosis that remains to be explored. Thus, the mechanisms identified in
this study likely contribute to vascular remodeling in rapidly growing populations of patients at high risk for cardiovascular disease and have important implications for developing new therapeutic strategies to treat vascular diseases. Current aldosterone antagonists therapies are limited by off-target (glycogenastasia) and renal (hyperkalemia) side effects. Thus, understanding the mechanisms by which vascular MR activation contributes directly to vascular disease could identify novel targets, including the PI3F/VEGFR1 pathway, which could reap the vascular benefits of MR blockade without the systemic side effects.

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Disclosures
None.

References
Significance

This article describes a new mechanism for the detrimental vascular effects of the blood pressure–regulating hormone aldosterone and for the beneficial effects of mineralocorticoid receptor antagonist drugs. The results demonstrate a blood pressure–independent role for aldosterone in vascular remodeling that is directly mediated by mineralocorticoid receptor in the smooth muscle cells of the vasculature. It provides a mechanistic explanation by which aldosterone enhances vascular remodeling specifically in areas of endothelial damage by upregulating smooth muscle cell expression of type 1 vascular endothelial growth factor receptors at sites of injury and promoting local production of the type 1 vascular endothelial growth factor receptor ligand, placental growth factor. Finally, it identifies the smooth muscle cell-mineralocorticoid receptor/type 1 vascular endothelial growth factor receptor pathway as a target that prevents aldosterone-enhanced vascular smooth muscle cell proliferation after endothelial injury without affecting endothelial regrowth with potential implications for new therapies to prevent the detrimental sequelae of hypertension and other adverse vascular remodeling outcomes, such as vein graft failure, in-stent-restenosis, or transplant allograft vasculopathy.
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<sup>f/f</sup>) with SMA-Cre-ERT2 mice (smooth muscle actin promoter driving expression of the Cre-ERT2 recombinase that is activated by tamoxifen<sup>2</sup>), as previously described<sup>1</sup>. Male MR<sup>f/f</sup>/SMA-Cre-ERT<sup>2+</sup> (SMC-MR-KO) and MR<sup>f/f</sup>/SMA-Cre-ERT<sup>2−</sup> 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<sup>1</sup>.

Wire carotid injury model and Evan's blue staining: The wire injury protocol has been previously described in detail<sup>3, 4</sup>. 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<sup>3, 4</sup>. 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<sup>5</sup>. 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<sup>6–8</sup> 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. 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), VEGFR1 antibody (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). 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. 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. RNA was reverse transcribed and PIGF 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.

**PIGF Protein Assay:** Media was collected from mouse aortas after treatment with vehicle or aldosterone for 8 hours and PIGF levels were measured by Quantikine mouse PIGF2 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


Supplemental Table I:

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<td>113.3 1.6</td>
<td>77.4 2.5</td>
<td>26.2 0.7</td>
<td>111.8 7.9</td>
<td>87.0 9.6</td>
<td>1.9 0.51</td>
</tr>
<tr>
<td>aldosterone + VEGFR2 ab</td>
<td>C57Bl/6</td>
<td>27.2 0.6</td>
<td>88.9 3.1</td>
<td>62.8 3.0</td>
<td>27.8 0.6</td>
<td>109.7 3.5</td>
<td>88.4 5.1</td>
<td>8.4 2.1*</td>
</tr>
</tbody>
</table>

Supplemental Table I: Increased serum aldosterone levels in aldosterone-infused mice treated with control IgG, VEGFR1-blocking antibody (ab), or VEGFR2-blocking ab with no change in blood pressure or animal weight. SBP=systolic blood pressure, DBP=diastolic blood pressure. * = P<0.05 vs vehicle.
Supplemental Figure I: Role of PIGF and VEGFR1 in aldosterone-enhanced carotid SMC proliferation in vitro.

(A) Primary mouse carotid SMC were treated in vitro with aldosterone (Aldo) at the indicated concentration and the percent increase in SMC proliferation compared to vehicle-treated cells was quantified after two days. N=6-7 experiments. *P<0.01 versus 0 nM Aldo.

(B) Mouse carotid SMC treated with vehicle, Aldo (10nM), or Aldo + eplerenone (EPL, 1µM) for 8 hours. PIGF and VEGFR1 mRNA expression were quantified by QRT-PCR. N=5-8 experiments. *P<0.05 versus vehicle.

(C) Mouse carotid SMC treated with Aldo (10nM) or vehicle (Veh) in the presence of control IgG, VEGFR1- or VEGFR2-blocking antibodies (ab) for 2 days. Bars represent the average percent increase in SMC proliferation compared to Veh with control IgG treatment. N=8 experiments. * P<0.05 versus all Veh-treated, # P<0.05 versus Aldo-treated with IgG.