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Aldosterone promotes vascular remodeling by direct effects on smooth muscle cell mineralocorticoid receptors

Dafina Pruthi[1], **Amy McCurley**[1], **Mark Aronovitz**[1], **Carol Galayda**[1], **S. Ananth Karumanchi**[2], and **Iris Z. Jaffe**[1]

[1]Molecular Cardiology Research Institute, Tufts Medical Center, Boston, MA

[2]Center for Vascular Biology Research, Beth Israel Deaconess Medical Center, Boston, MA

Abstract

Objective—Vascular remodeling occurs after 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 (PlGF)-dependent manner. We now test the hypothesis that SMC mineralocorticoid receptors (MR) 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 (WT) mice and mice with inducible SMC-specific deletion of MR (SMC-MR-KO). Aldosterone did not affect re-endothelialization after injury in WT 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-MR-KO mice. Further exploration of the mechanism revealed that aldosterone-induced vascular remodeling is prevented by blockade of the PlGF-specific receptor, VEGFR1, *in vivo*. Immunohistochemistry of carotid vessels shows that the induction of VEGFR1 expression in SMC after vascular injury is attenuated by 72% in SMC-MR-KO mice. Moreover, aldosterone induction of vascular PlGF 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 PlGF 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.

Keywords

vascular remodeling; smooth muscle cells; aldosterone; mineralocorticoid receptor; VEGF

Introduction

Vascular remodeling occurs in response to endothelial damage and contributes to vascular pathologies including vascular stiffness due to hypertension and aging, atherosclerosis, vein

Correspondence to: Iris Z. Jaffe, M.D./Ph.D., Tufts Medical Center, Molecular Cardiology Research Institute, 800 Washington St, Box 80, Boston, MA 02111. ijaffe@tuftsmedicalcenter.org, Phone 617-636-0620, Fax 617-636-1444.

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graft failure, restenosis after percutaneous vascular procedures, and cardiac transplant vasculopathy (reviewed in [1]). Endothelial damage can be caused by mechanical injury or by cardiovascular risk factors including dyslipidemia, hypertension, diabetes, or smoking. In the area of endothelial damage the normally quiescent smooth muscle cells (SMC) proliferate and produce extracellular matrix that contributes to vascular thickening and fibrosis. Although much has been learned about mechanisms of vascular remodeling, our current cardiovascular therapies are still limited by adverse remodeling that contributes to myocardial infarction (MI), stroke and the high failure rate of vein grafts, transplants and even stents. Thus there remains a need to identify novel contributors to vascular remodeling that might be more effective targets to prevent adverse cardiovascular events and improve the efficacy of our interventions.

Aldosterone is a steroid hormone that regulates blood pressure (BP) by acting on renal mineralocorticoid receptors (MR) to induce genes in the kidney that promote sodium retention[2]. MR antagonists, including spironolactone and eplerenone, are used to treat hypertension and heart failure and significantly reduce cardiovascular mortality in randomized trials[3–6]. Additional clinical data suggest that aldosterone promotes atherosclerotic ischemic events including MI and stroke and increases mortality[7,8]. In animal models of vascular injury, aldosterone enhances vascular remodeling[9,10]. Conversely, MR antagonists decrease vascular remodeling in animal models of hypertension[11], balloon injury[10], stent implantation[12], vein grafting[13], and hyperlipidemia-induced atherosclerosis[14]. Thus aldosterone plays an important role in vascular remodeling that has largely been attributed to BP elevation with secondary vascular consequences. However, it has recently become clear that aldosterone also has extra-renal actions. MR is expressed in vascular SMC and endothelial cells (EC) where it regulates genes involved in vascular inflammation, fibrosis and calcification[15–19]. Indeed we have recently demonstrated in a mouse model with inducible, SMC-specific MR deletion, that SMC-MR directly contributes to vascular contractile function and BP elevation with aging[20]. In humans, the cardiovascular protective effects of MR antagonists exceed the expected effects of modest changes in systemic BP[3,4,21]. These clinical and experimental findings support the possibility that aldosterone could act directly on MR in the vasculature to contribute to vascular remodeling. If so, understanding the vascular cellular target that mediates aldosterone-enhanced remodeling (EC versus SMC) and the potential molecular downstream mechanisms could provide novel therapeutic strategies.

The vascular endothelial growth factors (VEGF) are a family of secreted proteins that contribute to angiogenesis. VEGFs modulate vascular SMC and EC cell function via transmembrane VEGF type 1 and type 2 receptors (VEGFR1 and VEGFR2)[22]. We previously discovered that aldosterone specifically regulates vascular expression of the VEGF-family member, placental growth factor (PlGF)[9,18], and that PlGF is necessary for aldosterone-enhanced vascular remodeling[9]. PlGF specifically binds to VEGFR1 while other VEGFs signal through both receptors[22]. In injured mouse vessels and in human vessels with atherosclerotic disease, aldosterone further enhances PlGF expression and specifically upregulates VEGFR1[9]. These data identify PlGF/VEGFR1 as a potential mediator of aldosterone-enhanced vascular remodeling. In this study we test the hypothesis that SMC-MR directly mediates the remodeling effects of aldosterone on injured vessels and further explore the role of PlGF/VEGFR1 as a potential downstream mechanism and therapeutic target to prevent aldosterone-enhanced vascular remodeling *in vivo*.

Materials and Methods

See online methods supplement for details.

Mice and wire carotid injury model

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. 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 Tamoxifen daily and studies were performed 4 weeks later[20].One day prior to carotid injury, vehicle or aldosterone infusion pumps (240μg/kg/d) were inserted and a Bromodeoxyuridine (BrDU) infusion pump placed at the time of injury. Two weeks after injury, BrDU positive cells, medial area and extracellular matrix quantified histologically by treatment- and genotype-blinded investigators and re-endothelialization was assessed by staining with Evans Blue dye. Mice were injected intraperitoneally with VEGFR1- or VEGFR2-blocking antibody at 35 mg/kg diluted in phosphate buffered saline (PBS) 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) and the data from these 2 control groups was pooled and referred to as “control IgG”.

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.

Results

Aldosterone does not alter the rate of re-endothelialization following 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 blood pressure[9]. It has been suggested that the rate of endothelial re-growth after arterial injury determines the degree of vascular remodeling with accelerated re-endothelialization leading to an attenuated injury response[23]. 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 prior to 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 injured carotid arteries immediately after the initial injury (day 0) and 1, 2, 3, 7, and 14 days after injury are shown in Figure 1B. Evans blue staining confirms complete denudation of the endothelium on day 0. Complete re-endothelialization of the artery is confirmed14 days after injury. Quantification of the residual denuded area reveals no significant difference in the percentage of area covered with endothelium in arteries from aldosterone compared to vehicle treated mice at all time points after injury (Figure 1C). These results suggest that aldosterone is not enhancing the vascular remodeling response by altering endothelial cell proliferation or migration and may instead be acting on MR elsewhere in the vessel so we next focused on the smooth muscle cells.

Aldosterone-enhances vascular injury by direct, blood pressure-independent, effects on SMC-MR

The role of SMC-MR in aldosterone-stimulated vascular injury was directly examined using a mouse model with MR genetically deleted in adulthood specifically from SMC (SMC-MR-KO) compared with MR Intact littermate controls[20]. Prior studies reveal that at 3-months of age, 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[20]. This is confirmed by tail cuff plethysmography in the specific mice used for carotid injury that cannot have concurrent telemetry (Table 1). Mice underwent the carotid injury protocol (Figure 1A) with insertion of a bromodeoxyuridine (BrDU) 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 with no effect on systolic BP or body weight (Table 1). In uninjured vessels there is minimal SMC proliferation, as measured by medial BrDU 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 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[9]. 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 physiologic and pathologic 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 SMC 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 PlGF[9] that binds specifically to VEGFR1. To explore the role of VEGF receptors in aldosterone-induced 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 (Supplemental Table I). 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*.

Since 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 SMC in a dose-dependent manner (Supplemental Figure IA) as it does for other cultured SMC[24]. However, at physiologically (1 nM) and pathologically (5–10 nM) 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 SMC results in a non-significant trend towards increased PlGF expression (much less than the 300% increase in whole vessels ([9] and Figure 5A)) and only a modest but significant increase in VEGFR1 expression that is prevented by co-treatment

with the MR-specific antagonist eplerenone (Supplemental Figure IB). *In vitro*, VEGFR1-blockade inhibits the modest aldosterone-enhanced SMC proliferation while VEGFR2-blockade prevents a significant aldosterone-induced increase in proliferation but is not significantly decreased compared to aldosterone with IgG (Supplemental Figure IC). 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 SMC after vascular injury

In healthy vessels, VEGFR1 is expressed exclusively in the endothelium however, upon vascular injury, VEGFR1 expression is activated on vascular SMC 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 while VEGFR1 is expressed robustly on EC and SMC in injured vessels even without exogenous aldosterone administration (Figure 4A). Serial sections incubated with CD31 (an EC marker) and SMC alpha actin (a SMC marker) are included in Figure 4A to confirm the identity of each cell type in the vessel.

To explore if 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 SMC 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 more than 3 fold (Figure 5) as previously demonstrated in WT 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 VEGFR1at 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 effecting endothelial re-growth. These adverse effects of the hormone are completely lost in mice with the MR specifically deleted from SMC 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 SMC 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[9, 18, 25] 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[26], congestive heart failure[27], hyperlipidemia[28], or localized vascular injury[9]. In healthy vessels with an intact endothelium, VEGFR1 is expressed only on EC and aldosterone activation of SMC-MR only modestly increases PlGF transcription and release[9] with no effect on SMC proliferation and fibrosis as PlGF receptors are not expressed on SMC under these conditions (Figure 6, left). Thus in healthy vessels, aldosterone does not disrupt the quiescent SMC phenotype and vessels do not undergo adverse remodeling even when aldosterone levels are high (for example 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[9, 18] and to the expression of VEGFR1 on SMC resulting in SMC proliferation and vascular fibrosis[25, 29]. Conversely, PlGF deficiency[9] or VEGFR1 blockade prevents aldosterone-enhanced remodeling after injury in mice. This data from mouse models is consistent human data demonstrating that aldosterone enhances PlGF and VEGFR1 expression in vessels from patients with severe atherosclerosis (undergoing coronary artery bypass grafting) but not in healthy human vessels (transplant donors)[9]. Based on these data, we propose the model in Figure 6 (right) that in diseased or injured vessel, SMC-MR locally enhances PlGF release and SMC VEGFR1 expression resulting in enhanced SMC proliferation, vascular fibrosis, and vessel thickening after injury. By this mechanism, aldosterone and SMC-MR promote adverse vascular remodeling that contributes to luminal narrowing and vascular stiffness, important contributors to cardiovascular disease. Treatment of diseased human vessels with the MR antagonist spironolactone ex vivo suppresses PlGF production[9] thus inhibition of this mechanism may contribute to the beneficial effects of MR antagonists in cardiovascular patients[3, 4, 6]. Specific inhibition of VEGFR1 prevented aldosterone-enhanced remodeling in vivo supporting the concept that SMC-MR-regulated pathways could be targeted to prevent or treat cardiovascular diseases.

For over 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 SMC. 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[20] 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 SMC 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[10] 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 since the aldosterone-enhanced mechanism of vascular remodeling identified here appears to dissociate SMC proliferation from endothelial re-growth, 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[30] 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. Telemetric monitoring is the gold standard for BP measurements in rodents however, since 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 to MR Intact controls at baseline or with aldosterone infusion[20]. Here we confirm by tail cuff plethysmography that the substantial changes in arterial remodeling responses are not due to large changes in BP although small BP differences (5–10 mmHg or less) cannot be accurately distinguished by this technique. Finally, understanding how the downstream signaling of MR and VEGFR1 converge in SMC cells to coordinate the proliferative response to injury is an important area for future investigation. Indeed, both MR[24] and VEGFR1[25] signaling have been shown to promote SMC proliferation by activation of MAP-kinase signaling *in vitro*. Since 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 SMC in areas of vascular injury. This mechanism is independent of alterations in endothelial re-growth, renal MR activation, and systemic BP. Clinically, elevated serum aldosterone is increasingly common due to an association with resistant hypertension[31], heart failure, and obesity[32] and aldosterone levels correlate with increased risk of MI, stroke and death[7]. 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 (gynecomastia) 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 PlGF/VEGFR1 pathway, which could reap the vascular benefits of MR blockade without the systemic side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard abbreviations

BP	Blood pressure
BrDU	bromodeoxyuridine
EC	endothelial cells
ECM	extracellular matrix
KO	knock out mouse
MR	Mineralocorticoid Receptor
PBS	phosphate buffered saline
PlGF	Placental Growth Factor
SMC	smooth muscle cells
SMC-MR-KO	smooth muscle cell-specific MR knockout mouse
VEGF	vascular endothelial growth factor
VEGFR1	type 1 vascular endothelial growth factor receptor
VEGFR2	type 2 vascular endothelial growth factor receptor
WT	wild type

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Significance

This manuscript describes a new mechanism for the detrimental vascular effects of the blood pressure regulating hormone aldosterone and for the beneficial effects of mineralocorticoid receptor (MR) antagonist drugs. The results demonstrate a blood-pressure independent role for aldosterone in vascular remodeling that is directly mediated by MR 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 SMC expression of type 1 vascular endothelial growth factor receptors (VEGFR1) at sites of injury and promoting local production of the VEGFR1 ligand, PlGF. Finally, it identifies the SMC-MR/VEGFR1 pathway as a target that prevents aldosterone-enhanced vascular SMC proliferation after endothelial injury without affecting endothelial re-growth 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.

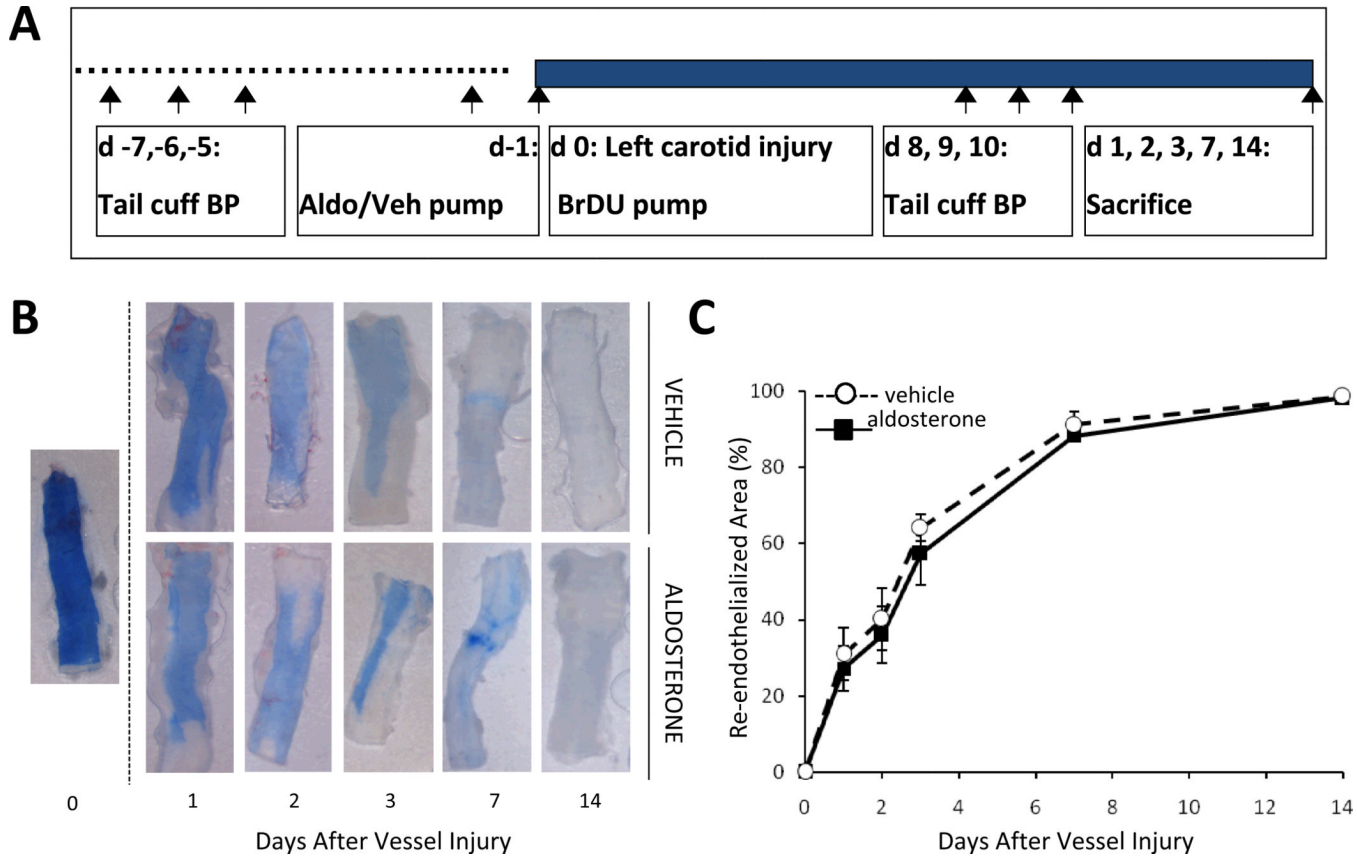

Figure 1. Aldosterone-enhanced vascular injury is independent of effects on endothelial re-growth

(A) Schematic of the mouse wire carotid injury model used for all *in vivo* studies. Mice were implanted with vehicle (Veh) or aldosterone (Aldo) infusion pumps one day prior to carotid denudation by wire injury. Tail cuff blood pressure (BP) measurements were conducted prior to and after injury. Mice were sacrificed at day 14 after injury unless otherwise indicated. (B) Representative carotid arteries showing Evan's Blue dye marking in blue the denuded area at the indicated times following carotid injury in C57Bl/6 mice. (C) Quantification of re-endothelialization calculated as the percent of the carotid area without Evan's Blue staining. N=3–5 mice/treatment/time. There is no significant difference between vehicle and aldosterone treated vessels.

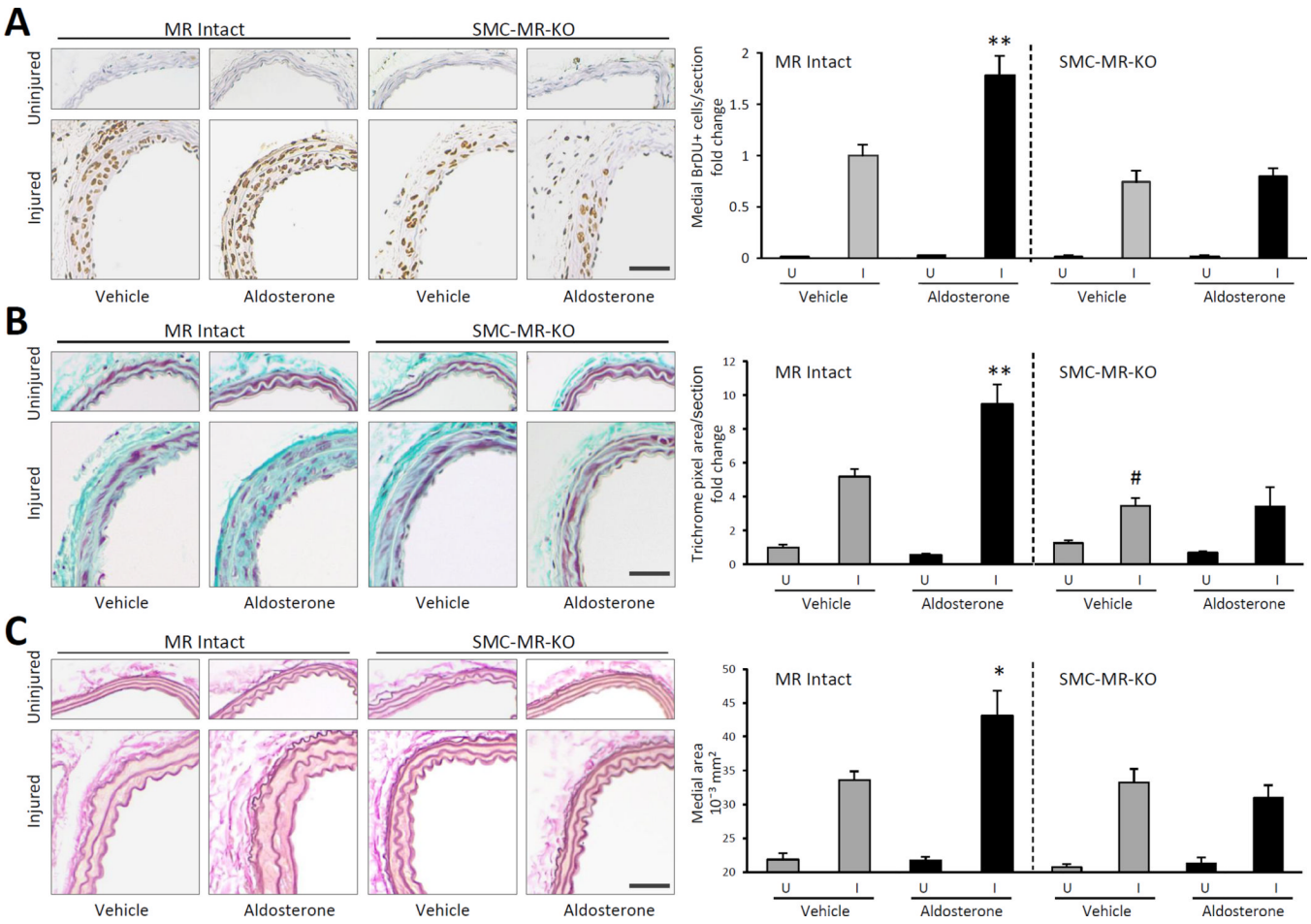

Figure 2. Smooth muscle cell MR is necessary for aldosterone-enhanced vascular remodeling after injury *in vivo*

Mice with MR specifically deleted from SMC (SMC-MR-KO) and MR intact littermates (MR Intact) underwent wire carotid injury along with two weeks of vehicle or aldosterone treatment. **(A)** Medial SMC proliferation was quantified in BrDU-stained sections of uninjured (U) and injured (I) mouse carotid arteries. Representative carotid artery sections are shown on left and the fold change in the number of medial BrDU positive SMC per section compared with MR Intact, vehicle-treated, injured vessels is indicated on right. **(B)** Fibrosis was quantified in trichrome-stained sections of uninjured and injured mouse carotid arteries. Representative carotid artery sections are shown on left and the fold change in medial trichrome pixel area compared with MR Intact, vehicle-treated, uninjured vessels is indicated on right. **(C)** Medial vessel area was quantified in Elastin-stained sections of uninjured and injured mouse carotid arteries treated with vehicle or aldosterone. Representative carotid artery sections are shown on left and the average medial area for all animals is indicated on right. Scale bar: 0.5mm. N=14–16 mice/genotype and treatment. *P < 0.01, **P < 0.001 versus all other injured vessels; # P<0.05 versus MR Intact, vehicle-treated, injured vessels.

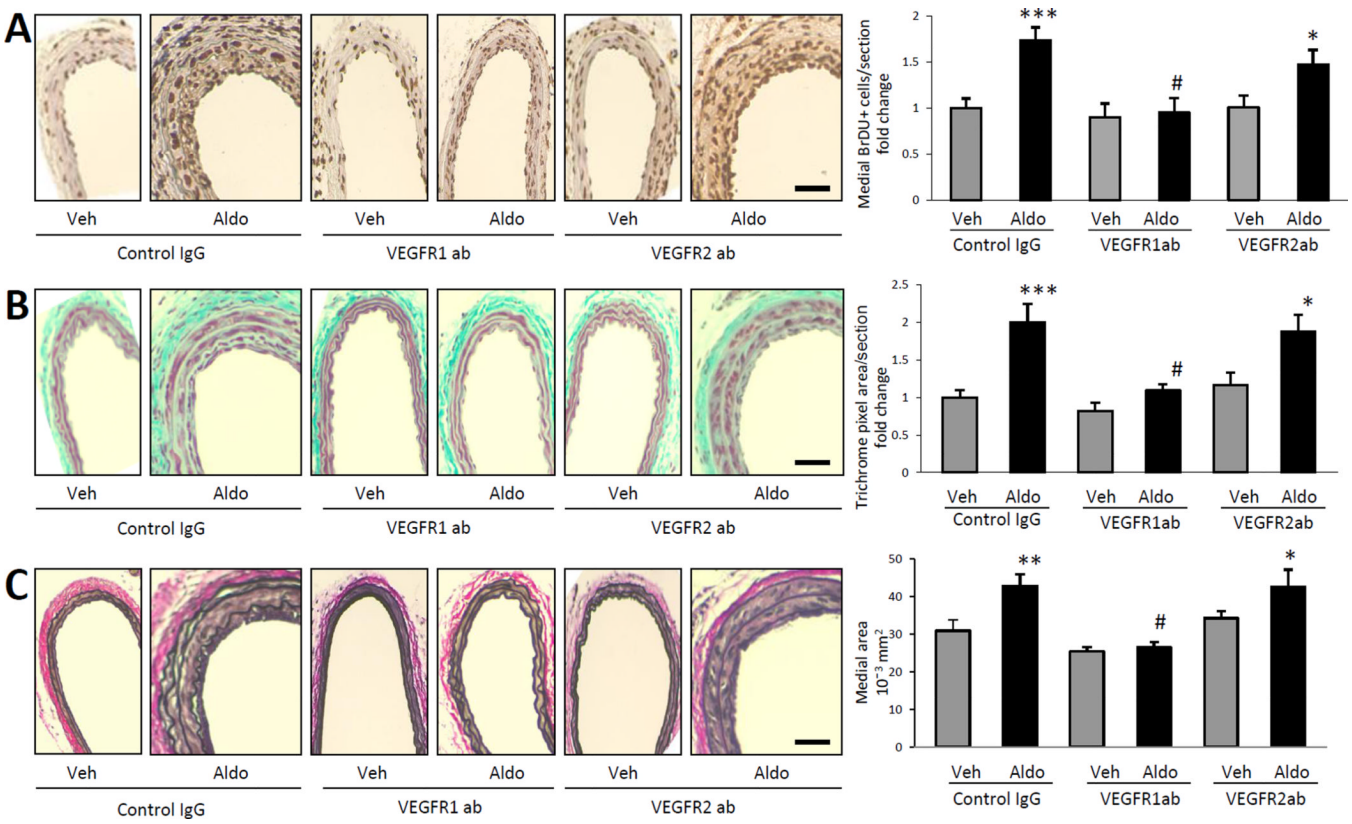

Figure 3. VEGFR1 blockade prevents aldosterone-enhanced vascular remodeling after injury

Mice underwent wire carotid injury along with vehicle (Veh) or aldosterone (Aldo) infusion and injection of control IgG, VEGFR1- or VEGFR2-blocking antibody (ab). **(A)** Medial smooth muscle proliferation (SMC) proliferation was quantified in BrDU-stained sections of injured mouse carotid arteries. Representative carotid artery sections are shown on the left. Bars on the right represent fold change in medial BrDU+ SMC relative to Veh with Control ab treatment. **(B)** Extra-cellular matrix deposition was quantified in trichrome-stained sections of injured mouse carotid arteries. Representative carotid artery sections are shown on the left. Bars on the right represent the fold change in medial trichrome pixel area relative to Veh with control IgG. **(C)** Medial vessel area was quantified in elastin-stained sections of injured mouse carotid arteries. Representative carotid artery sections are shown on left. Bars on the right represent average medial area for all animals. Scale bar: 0.5mm. N=8–12. *P<0.05, **P<0.01, ***P<0.001 versus all Veh-treated. #P<0.01 versus Aldo with Control IgG and Aldo with VEGFR2ab treatment.

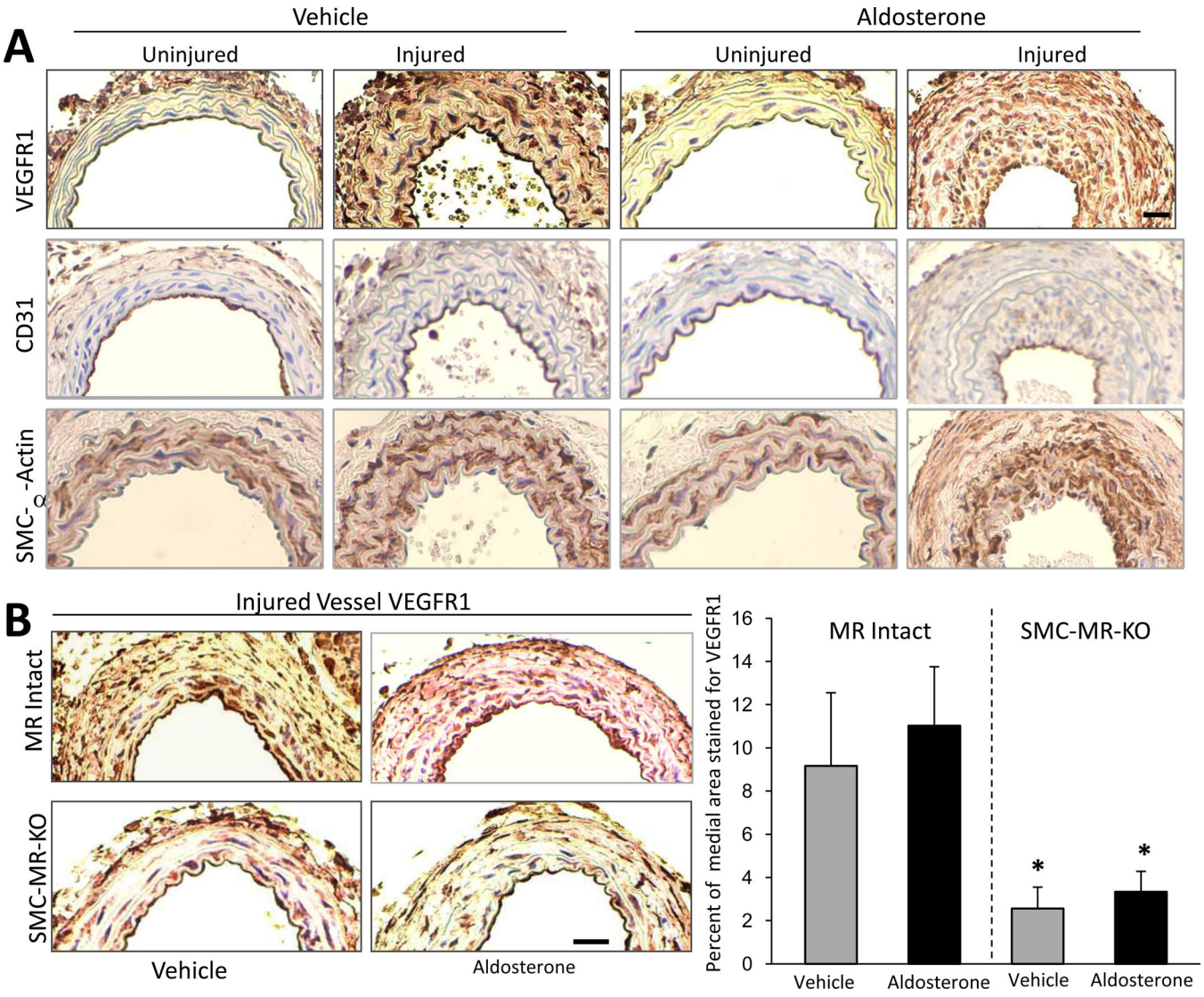

Figure 4. SMC-MR contributes to injury-induced SMC VEGFR1 expression

(A) VEGFR1 is expressed exclusively on EC in uninjured vessels and is induced on SMC after vascular injury. Immunohistochemistry of serial carotid artery sections 14 days after unilateral carotid injury of wild type mice using VEGFR1 antibody to localize expression and anti CD31 antibody to label endothelial cells and anti smooth muscle α-actin antibody to label SMC. **(B)** SMC-MR contributes to induction of SMC VEGFR1 expression after endothelial injury. VEGFR1 immunohistochemistry of sections of injured carotids from MR-Intact and SMC-MR-KO littermates 14 days after injury. Representative carotid artery sections are shown on the left and the percent of the medial area that stains positive for VEGFR1 is quantified on the right. Scale bar: 0.5mm. N=8. *P<0.05 versus MR Intact vehicle- and aldosterone-treated vessels.

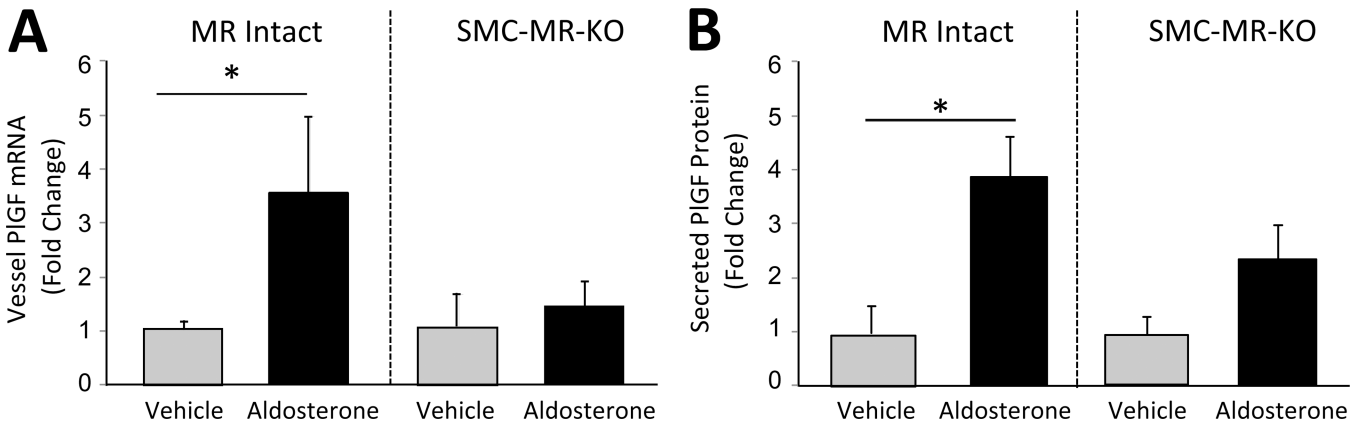

Figure 5. SMC-MR is necessary for aldosterone regulation of vascular PlGF expression and release

(A,B) Mouse aortas from 3-month old MR Intact and SMC-MR-KO mice were treated for 8 hours *ex vivo* with vehicle (grey bars) or 100nM aldosterone (black bars). **(A)** PlGF mRNA expression was quantified by QRT-PCR of vessel RNA and **(B)** PlGF protein secretion was quantified by ELISA of vessel conditioned media. *P < 0.05 versus vehicle.

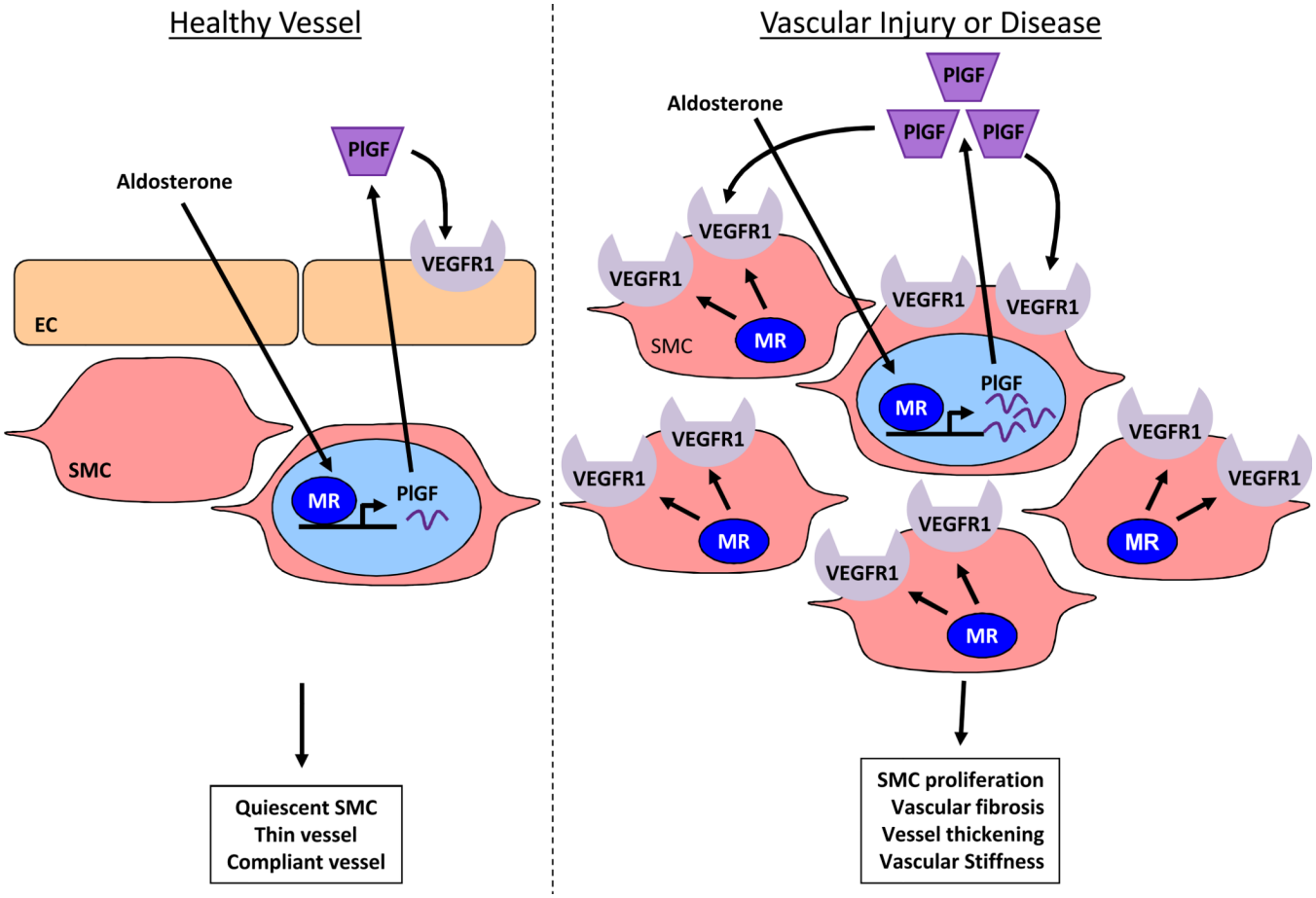

Figure 6. Model for the role of the SMC-MR/PlGF/VEGFR1 pathway in aldosterone-induced vascular remodeling after injury

In the healthy uninjured vasculature (left), vascular endothelial growth factor type 1 receptors (VEGFR1) are expressed exclusively on endothelial cell (EC) and not on smooth muscle cells (SMC). Aldosterone activation of SMC mineralocorticoid receptors (MR) in this setting modestly enhances placental growth factor (PlGF) transcription and local release that can act only on EC expressing VEGFR1. Thus in healthy vessels, aldosterone does not disrupt the quiescent SMC phenotype and vessels do not undergo adverse remodeling even when aldosterone levels are high. In the setting of vascular injury or disease (right), SMC-MR contributes to local upregulation of VEGFR1 expression on SMC and aldosterone activation of SMC-MR contributes to substantial PlGF expression and release. PlGF mediates aldosterone-enhanced SMC proliferation and vascular fibrosis after injury by binding to VEGFR1 receptors on SMC. This model provides insight into how aldosterone acts synergistically with endothelial injury to contribute to adverse vascular remodeling and how MR antagonists prevent adverse cardiovascular events in clinical trials.

Table 1

Increased serum aldosterone to pathological levels in aldosterone-infused MR intact and SMC-MR-KO mice with no change in blood pressure or animal weight.

Treatment	Strain	pre-treatment weight (g)	pre-treatment SBP (mmHg)	pre-treatment DBP (mmHg)	pre-harvest weight (g)	treatment SBP (mmHg)	treatment DBP (mmHg)	serum aldosterone (nM)
vehicle	MR Intact	27.3 ± 1.2	109.5 ± 3.4	81.7 ± 3.8	28.0 ± 1.0	115.7 ± 2.5	85.9 ± 2.8	1.2 ± 0.14
aldosterone	MR Intact	26.9 ± 0.7	104.8 ± 3.0	76.2 ± 3.3	27.8 ± 0.7	107.1 ± 2.4	80.1 ± 2.7	7.6 ± 0.71*
vehicle	SMC-MR-KO	27.4 ± 0.8	104.3 ± 3.1	76.7 ± 3.1	28.2 ± 0.7	109.1 ± 4.1	80.5 ± 3.9	1.3 ± 0.11
aldosterone	SMC-MR-KO	27.3 ± 0.6	106.4 ± 3.3	77.5 ± 3.3	28.1 ± 0.5	110.0 ± 3.0	81.0 ± 2.8	6.5 ± 0.74*

SBP=systolic blood pressure, DBP=diastolic blood pressure.

* P<0.001 versus vehicle.

g=grams.