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Placental growth factor mediates aldosterone-dependent vascular injury in mice

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In clinical trials, aldosterone antagonists reduce cardiovascular ischemia and mortality by unknown mechanisms. Aldosterone is a steroid hormone that signals through renal mineralocorticoid receptors (MRs) to regulate blood pressure. MRs are expressed and regulate gene transcription in human vascular cells, suggesting that aldosterone might have direct vascular effects. Using gene expression profiling, we identify the pro-proliferative VEGF family member placental growth factor (PGF) as an aldosterone-regulated vascular MR target gene in mice and humans. Aldosterone-activated vascular MR stimulated *Pgf* gene transcription and increased PGF protein expression and secretion in the mouse vasculature. In mouse vessels with endothelial damage and human vessels from patients with atherosclerosis, aldosterone enhanced expression of PGF and its receptor, FMS-like tyrosine kinase 1 (Flt1). In atherosclerotic human vessels, MR antagonists inhibited PGF expression. In vivo, aldosterone infusion augmented vascular remodeling in mouse carotids following wire injury, an effect that was lost in *Pgf*^{-/-} mice. In summary, we have identified PGF as what we believe to be a novel downstream target of vascular MR that mediates aldosterone augmentation of vascular injury. These findings suggest a non-renal mechanism for the vascular protective effects of aldosterone antagonists in humans and support targeting the vascular aldosterone/MR/PGF/Flt1 pathway as a therapeutic strategy for ischemic cardiovascular disease.

Introduction

In heart failure trials, in addition to reducing cardiovascular mortality, aldosterone antagonists have also been unexpectedly shown to significantly reduce atherosclerotic clinical events by mechanisms that remain unclear (1, 2). More recently, multiple large clinical trials of the CETP inhibitor torcetrapib were terminated early due to increased cardiovascular mortality despite very favorable lipid profiles (3–5), likely due to off-target elevations of aldosterone stimulated by the drug (6–9). These data from randomized clinical trials support the idea that aldosterone promotes atherosclerotic cardiovascular events and increases mortality in humans, and that these adverse effects are inhibited by aldosterone antagonists. These clinical data underscore the need to explore the molecular mechanisms of aldosterone-mediated vascular disease, both to explain these important clinical findings and to identify potential novel therapeutic targets for common vascular diseases.

Aldosterone is a steroid hormone that acts by binding to the mineralocorticoid receptor (MR, or Nr3c2), a member of the nuclear hormone receptor family of ligand-activated transcription factors. Aldosterone elevates systemic blood pressure by activating renal MR-dependent gene transcription (10), and aldosterone-induced increases in cardiovascular events have previously been ascribed primarily to renal MR-mediated blood pressure elevation with secondary vascular consequences (11). However, the reduction of cardiovascular events in clinical trials of aldosterone antagonists is significantly greater than that expected from the modest decrease in systemic blood pressure in treated patients (1, 2). These observations support the hypothesis that non-renal aldosterone/MR effects contribute importantly to cardiovascular pathology.

Vascular remodeling is a pathologic response of the vessel to vascular damage and contributes to human ischemic vascular disease. Remodeling occurs when endothelial cells (ECs) are damaged by insults from traditional cardiac risk factors such as cigarette smoke toxins, diabetes, hypertension, and dyslipidemia and by mechanical injury. Endothelial damage initiates a cascade of events that constitute the vascular injury response, resulting in activation of SMCs to migrate, proliferate, and produce ECM (reviewed in ref. 12). When these physiologic processes are excessive, for example in restenosis following percutaneous vascular procedures or in chronic atherosclerotic vascular disease, the resulting intimal and medial hyperplasia compromises the vascular lumen and blood flow to distal tissues. Animal studies in rat, rabbit, and pig models demonstrate that aldosterone infusion in vivo enhances vascular remodeling in response to endothelial damage or dysfunction (13–15). The vascular effects of aldosterone are reversed by aldosterone antagonists, implicating MR in the mechanism (16), although the role of vascular versus renal MR in these models is unclear. In humans, increased carotid artery thickening is associated with electrolyte evidence of aldosterone excess (7), and inhibition of the angiotensin-aldosterone pathway slows progression of carotid remodeling (17).

Over the past decade, evidence has accumulated for the presence of functional MR expression in cardiovascular tissues (18). We recently demonstrated that human VSMCs (19) and ECs (20) express MR that is capable of regulating the vascular-specific gene expression programs involved in vascular fibrosis and calcification (19, 21), oxidative stress (20), and cell adhesion (20). These in vitro data demonstrate that vascular cells contain functional MR capable of responding directly to aldosterone. However, direct MR target genes in intact blood vessels have not been systematically identified, nor has the potential for such genes to mediate aldosterone-stimulated vascular remodeling in vivo been adequately explored.

Conflict of interest: Michael E. Mendelsohn is employed by Merck but retains an academic appointment at Tufts University School of Medicine.

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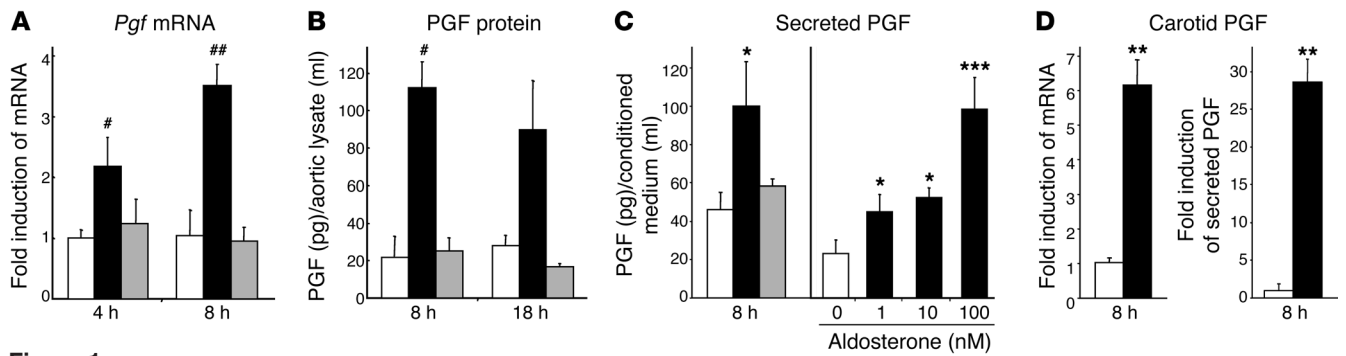


Figure 1

PGF expression is regulated by aldosterone via vascular MR activation. Mouse aortas (A–C) or carotid arteries (D) were treated ex vivo with vehicle (white bars), aldosterone (black bars, 100 nM unless otherwise indicated), or aldosterone + spironolactone (gray bars, 10 μM spironolactone) for the indicated times. (A–C) *Pgf* mRNA expression was quantified by QRT-PCR of vessel RNA (A), *Pgf* protein was quantified by ELISA in vessel lysate (B), and secretion was quantified by ELISA of vessel conditioned media (C). (D) Carotid *Pgf* mRNA (left) and secreted protein (right) were quantified. #*P* < 0.05, ##*P* < 0.001 versus vehicle and aldosterone + spironolactone. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus vehicle.

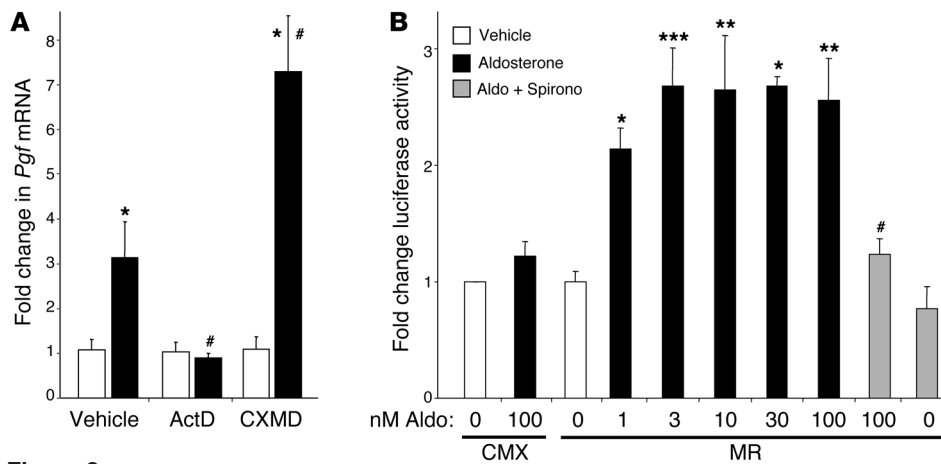
Placental growth factor (PGF) is a secreted peptide member of the VEGF family that is known to promote vascular cell proliferation and vascular inflammation by binding specifically to the transmembrane receptor, fms-like tyrosine kinase 1 (Flt1, or VEGFR-1). PGF has been implicated in vascular remodeling, atherosclerosis, and adverse ischemic events in animal models and in humans (22–26). In this study, we identify PGF as what we believe to be a novel target of vascular MR activation. We demonstrate here that *PGF* is a vascular MR-regulated gene, we explore the mechanism of PGF regulation by MR, and we show that PGF deficiency prevents aldosterone-mediated vascular remodeling in vivo. These findings support the idea that PGF is a key mediator of the adverse vascular effects of aldosterone and help to identify the PGF pathway as a potentially novel drug target for ischemic cardiovascular diseases.

Results

Aldosterone promotes vascular remodeling in vivo. VSMC proliferation and vascular fibrosis are two important components of the vascular injury response that have been shown to be influenced by aldosterone in non-murine animal models (13). As a first step toward understanding the mechanisms that mediate the adverse vascular effects of aldosterone, we began by exploring the effect of aldosterone in the mouse wire carotid injury model, a reproducible method to study these specific responses to endothelial injury (27–32). Aldosterone was infused at a dose that increased circulating aldosterone concentrations to levels consistent with what is found in patients with heart failure and atherosclerosis (33, 34) without altering systolic blood pressure or animal weight (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI40205DS1). Vascular injury resulted in increased medial SMC proliferation, which was significantly augmented by low-dose aldosterone infusion (Supplemental Figure 1A). Aldosterone also significantly enhanced wire injury-induced increases in ECM deposition and vascular medial area (Supplemental Figure 1, B and C, respectively). Notably, aldosterone infusion alone, in the absence of vascular injury, had no significant effect on medial SMC proliferation, medial ECM, or vascular medial area (uninjured vessels in Supplemental Figure 1). These data suggest that aldosterone, at pathologically relevant concentrations that do not increase blood pressure, acts synergistically with mechanical endothelial damage to promote vascular remodeling in vivo in mice.

Vascular PGF expression is regulated by aldosterone via vascular MRs. We have shown previously that MR is expressed in human vessels and regulates gene transcription in vascular cells (19, 20). We hypothesized that MR-regulated genes in blood vessels may play a role in the mechanism of aldosterone-enhanced vascular injury. We carried out extensive expression profiling of RNA from aldosterone-stimulated mouse aortas and identified substantial induction of PGF by aldosterone in these ex vivo vascular expression studies (1.7-fold, 4.6-fold, and 4.6-fold increase with aldosterone- compared with vehicle-treated vessels after 2, 4, and 8 hours of aldosterone treatment respectively; NCBI GEO accession #GSE23566). We pursued this aldosterone-regulated VEGF family member because of the potential pathophysiological significance of the known role of the VEGF family in vascular cell proliferation. We confirmed aldosterone-mediated increases in *Pgf* mRNA in isolated mouse aortas (Figure 1A) and observed increases in vascular PGF protein and extravascular PGF secretion after aldosterone treatment (Figure 1, B and C). We found that aldosterone regulation of vascular PGF expression and secretion occurred at physiologic (1 nM) and pathologic (2–10 nM) aldosterone concentrations (Figure 1C and data not shown). Aldosterone-mediated increases in aortic PGF message, protein, and secretion were all inhibited by ex vivo vessel treatment with the MR antagonist spironolactone (Figure 1, A–C), which supports the idea that aldosterone regulation of PGF is mediated by vascular MR. Given recent reports suggesting that distinct vascular beds respond differently to stimuli (35), we also tested the effects of aldosterone on carotid artery PGF expression. Aldosterone significantly enhanced *Pgf* mRNA expression and protein secretion from mouse carotid vessels to an extent significantly greater than that observed in the aorta (28.6-fold ± 3.0-fold vs. 2.8-fold ± 0.3-fold; *P* = 0.001; see also Figure 1D), suggesting that PGF regulation by aldosterone may play a role in local aldosterone-stimulated vascular remodeling in the carotid wire injury model. Aldosterone had no significant effect on expression of the VEGF receptors Flt1 or Flk1 (Vegfr-2) in the intact mouse carotid artery (data not shown).

*Aldosterone regulates PGF transcription and activates an MR response element upstream of the *Pgf* gene.* The aldosterone-stimulated increase in aortic *Pgf* mRNA was completely inhibited by pretreatment with the transcription inhibitor actinomycin D (Figure 2A), indicating

**Figure 2**

Mechanism of aldosterone regulation of PGF expression. (A) Aldosterone regulates PGF transcription. Mouse aortas were treated ex vivo with vehicle (white bars) or aldosterone (black bars) in the presence of vehicle, 5 $\mu\text{g/ml}$ actinomycin D (ActD), or 40 $\mu\text{g/ml}$ CXMD, and *Pgf* mRNA expression was quantified by QRT-PCR of vessel RNA. * $P < 0.001$ versus vehicle, # $P < 0.05$ versus aldosterone treated with no inhibitor. (B) The *PGF* gene contains a functional MRE. HEK293 cells were transfected with a luciferase reporter driven by the PGF enhancer MRE in the absence (empty CMX vector) or presence of MR expression. The cells were treated with the indicated concentrations of aldosterone and/or 10 μM spironolactone (gray bars). Bars represent fold activation of normalized luciferase activity relative to vehicle-treated, similarly transfected cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus MR plus 0 nM aldosterone and versus spironolactone alone. # $P < 0.01$ versus 100 nM aldosterone.

that aldosterone-induced increases in PGF are due to MR-dependent increases in *Pgf* gene transcription. The aldosterone effect was not inhibited by pretreatment with the translation inhibitor cyclohexamide (CXMD), suggesting that the transcription factors and cofactors required for aldosterone-mediated PGF expression are present in the vessel at the time of aldosterone exposure.

To identify potential MR response elements (MREs) that may mediate MR regulation of PGF transcription, we searched the *Pgf* gene upstream region computationally for pairs of known MR-binding hexameric half-sites (36) spaced by up to 9 bases (as in the known Na/K ATPase gene; ref. 37) and identified a single potential MRE consisting of 2 perfectly matched and highly evolutionarily conserved MR half-sites spaced by 5 bases (the size, but not the sequence, of the spacer is also conserved in at least 7 mammalian species) (Supplemental Figure 2). When inserted upstream of a luciferase reporter and transfected into HEK293 cells along with the MR, this binding site mediated aldosterone-dependent gene transcription (Figure 2B). Aldosterone activation of this DNA element was dose dependent in the physiologic and pathologic range and was MR dependent, as demonstrated by the lack of aldosterone activation in the absence of cotransfected MR and by complete inhibition of the aldosterone effect with the MR antagonist spironolactone.

In injured vessels, aldosterone further enhances PGF expression and upregulates the PGF receptor *Flt1*. Aldosterone-stimulated PGF expression was measured ex vivo in intact mouse aortas and in aortas injured by endothelial denudation. The extent of aldosterone-enhanced *Pgf* mRNA expression (Figure 3A) and protein secretion (Figure 3B) were significantly increased in denuded vessels compared with uninjured aortas, indicating that the endothelium plays a protective role in aldosterone-stimulated vascular PGF expression as it does for the aldosterone-stimulated vascular injury in the in vivo wire injury model (Supplemental Figure 1). The finding of enhanced aldosterone regulation in injured vessels distinguished

PGF from most other vascular MR target genes tested (data not shown), further supporting the focus on PGF as a candidate to mediate the synergistic effects of aldosterone with vascular endothelial injury.

We also explored aldosterone regulation of other VEGF ligands and receptors in intact and injured vessels. Aldosterone specifically enhanced mRNA expression of the PGF-binding VEGF receptor, *Flt1*, in denuded, but not intact, vessels (Figure 3C). Aldosterone had no effect on expression of *Flk1* (the receptor for other VEGF family members; see ref. 38 and Figure 3C), nor did aldosterone alter the level of secreted VEGF in the medium in either intact or denuded vessels (data not shown), suggesting that aldosterone specifically enhances the PGF/*Flt1* pathway in the setting of vascular endothelial injury.

Vascular MR activation regulates PGF in diseased human vessels. Regulation of PGF by aldosterone was next examined in healthy and diseased human

vessels. *PGF* mRNA was significantly elevated in aortic tissue from patients with atherosclerotic vascular disease that required coronary artery bypass graft (CABG), compared with normal human aortic tissue obtained from cardiac transplant donors (Figure 4A), which suggests that PGF expression is increased in vessels from humans with atherosclerosis. In normal human vessels, aldosterone treatment modestly but not significantly increased PGF expression (1.64-fold \pm 0.36-fold; $P = 0.098$), but in atherosclerotic vessels aldosterone significantly increased PGF expression (2.7-fold \pm 0.5-fold; $P < 0.05$; Figure 4B) and *Flt1* expression (2.6-fold \pm 0.1-fold; $P < 0.01$; Figure 4B). To explore whether MR activation is driving PGF expression in diseased vessels under physiologic conditions, PGF mRNA expression and protein secretion were measured in atherosclerotic human vessels treated ex vivo with the MR antagonist spironolactone (1 μM , in serum containing medium). MR antagonism resulted in a significant 80% reduction in PGF mRNA expression and protein secretion (Figure 4, C and D, respectively). *FLT1* mRNA expression also tended to decrease with spironolactone treatment of diseased vessels, but this was not statistically significant (67% \pm 20% decrease; $P = 0.1$). These data support a potential role for aldosterone and vascular MR in regulating the PGF/*Flt1* pathway in humans with underlying atherosclerosis, a condition characterized by vascular endothelial dysfunction, and suggest that inhibition of this pathway could contribute to the beneficial effects of MR antagonists in human trials.

Diminished aldosterone-mediated augmentation of vascular injury in PGF-deficient mice. The role of PGF in aldosterone-stimulated vascular injury was next studied in mice genetically deficient in PGF compared with PGF-intact control mice. At baseline, PGF-deficient mice have a modest but significant elevation in systemic blood pressure compared with WT control mice (systolic blood pressure prior to randomization: 117.3 \pm 2.8 mmHg in WT vs. 127.4 \pm 3.4 mmHg in PGF KO; $P < 0.05$; Table 1). Aldosterone infusion increased circulat-

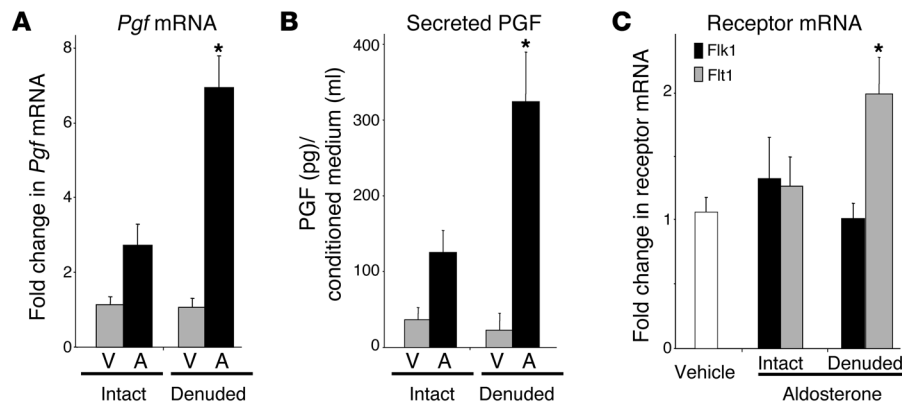


Figure 3 Endothelial injury enhances aldosterone-stimulated expression of PGF and its receptor, Flt1. Intact and endothelium-denuded mouse aortas were treated ex vivo with aldosterone (A) or vehicle (V) for 8 hours. (A) Mouse aortic *Pgfr* message was quantified by QRT-PCR. (B) Mouse *Pgfr* secretion was quantified by ELISA using vessel-conditioned media. (C) Mouse aortic Flt1 and Flk1 messages were quantified by QRT-PCR. **P* < 0.05 versus vehicle-treated and versus intact aldosterone-treated aortas.

ing aldosterone levels significantly and similarly in the two genotypes with no effect on systolic blood pressure or body weight in either genotype (Table 1). In WT mice, aldosterone had no effect on systemic serum PGF levels, and serum PGF was undetectable in PGF KO mice in the absence or presence of aldosterone infusion, and thus was significantly reduced in comparison to WT mice (Table 1).

In PGF intact mice on the FVB background, wire injury increased VSMC proliferation, as expected, and this effect was significantly enhanced by aldosterone (Figure 5), similar to the effect in WT C57BL/6 mice (Supplemental Figure 1A), confirming that the aldosterone effects on vascular remodeling occur on multiple mouse genetic backgrounds. Wire injury also increased VSMC proliferation in vehicle-treated PGF KO mice. However, injury-induced SMC proliferation in PGF KO mice was not significantly enhanced by aldosterone treatment (Figure 5). Furthermore, the level of SMC proliferation observed in injured, aldosterone-treated PGF KO mouse vessels was significantly lower than that observed in injured, aldosterone-treated WT mouse vessels. Aldosterone infusion significantly enhanced injury-induced medial collagen deposition in WT mice (Figure 6). However, in PGF-deficient mice, aldosterone infusion did not increase vascular medial ECM after carotid wire

injury (Figure 6), supporting the idea that PGF is necessary for aldosterone-mediated ECM induction in WT animals. Finally, wire vascular injury caused an increase in vessel medial area that was enhanced in the presence of aldosterone in WT but not PGF KO mice (Figure 7), and the magnitude of vessel thickening in aldosterone-treated PGF-deficient mice was significantly decreased when compared with aldosterone-treated WT mice. Taken together, these data suggest that PGF plays a significant role in the mechanism of aldosterone-enhanced vascular injury in this model.

Discussion

In summary, we have identified the VEGF family member *PGF* as what we believe is a novel vascular MR-regulated gene that contributes to aldosterone-mediated augmentation of vascular injury. Aldosterone-activated vascular MR stimulates PGF transcription and increases vascular PGF protein expression and secretion. In mouse vessels with endothelial damage and in human vessels from patients with atherosclerosis, aldosterone further enhances vascular expression of both PGF and of the specific PGF receptor, Flt1. Enhanced PGF expression in atherosclerotic human vessels is substantially inhibited by treatment with a clinically beneficial MR antagonist.

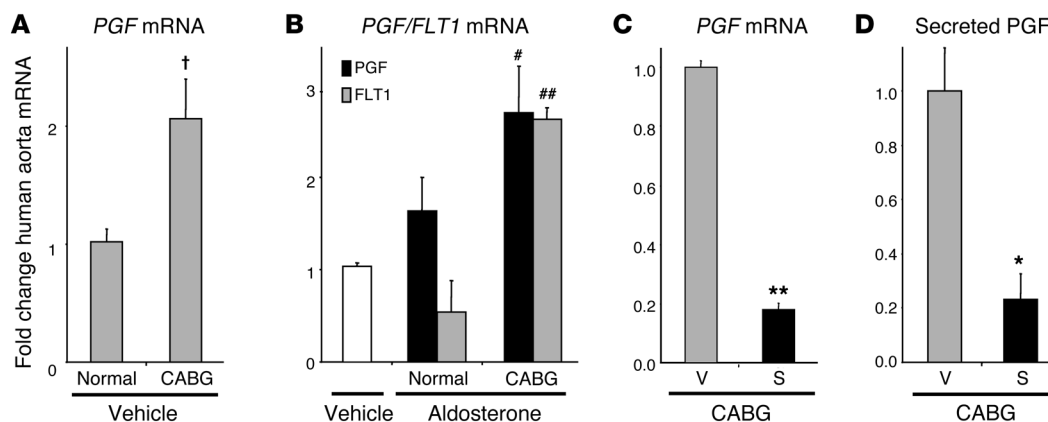


Figure 4 PGF regulation by aldosterone and MR in atherosclerotic human vessels. Normal and atherosclerotic human vessels (from CABG patients) were treated ex vivo with vehicle control (V), aldosterone (8 hours), or spironolactone (S) (1 μM, 18 hours). (A) Basal human PGF message was quantified by QRT-PCR. (B) Aldosterone induction of human aortic PGF and FLT1 messages were quantified by QRT-PCR. (C) Spironolactone effects on human PGF message was quantified by QRT-PCR. (D) Spironolactone effects on human PGF protein secretion was quantified by human PGF ELISA of vessel-conditioned media. †*P* < 0.05 versus normal vessel. #*P* < 0.05, ##*P* < 0.01 versus vehicle-treated and versus aldosterone-treated normal vessel mRNA. **P* < 0.05, ***P* < 0.001 versus vehicle-treated CABG vessel.



Table 1
WT and PGF KO mice animal characteristics

	WT		PGF KO		P value
	Vehicle	Aldosterone	Vehicle	Aldosterone	
Baseline					
Systolic BP (mmHg)	112.2 ± 3.7	122.3 ± 4.1	127.4 ± 4.9	127.3 ± 4.9	< 0.05 ^A
Weight (g)	25.6 ± 0.4	27.5 ± 0.9	29.5 ± 0.8	26.8 ± 2.1	NS
Day 14 after vehicle/aldosterone pump					
Systolic BP (mmHg)	118.8 ± 4.8	115.3 ± 3.3	132.9 ± 5.2	126.6 ± 8.0	0.06 ^A (no effect of hormone)
Weight (g)	28.5 ± 0.4	29.7 ± 0.4	28.4 ± 0.8	27.9 ± 0.9	NS
Serum aldosterone (nM)	0.7 ± 0.6	4.0 ± 0.6	0.9 ± 0.6	4.3 ± 0.6	< 0.001 ^B (no effect of genotype)
Serum PGF (pg/ml)	99.3 ± 29.5	53.3 ± 13.9	Undetectable	Undetectable	< 0.001 ^A (no effect of hormone)

^AWT compared with PGF KO. ^BVehicle compared with aldosterone.

Finally, in a mouse model of wire carotid injury, aldosterone infusion to levels found in patients with heart failure and atherosclerosis increases SMC proliferation, ECM deposition, and vascular medial area. In mice genetically deficient in PGF, aldosterone-mediated augmentation of vascular injury is prevented. Taken together, these data identify a role for PGF in aldosterone-stimulated vascular injury and provide a novel molecular mechanism for the protective effects of aldosterone antagonists in cardiovascular patients.

Several mRNAs and proteins, including monocyte chemoattractant protein 1 (MCP1), osteopontin, and TNF- α have been shown previously to be increased in perivascular and cardiovascular tissues after aldosterone infusion into animals (15, 39), though the

mechanisms mediating the increased expression of these molecules are not known and could include aldosterone activation of renal MR with secondary effects on vascular gene expression. This study examined whole vessels treated ex vivo with aldosterone to identify *PGF* as a novel aldosterone-induced vascular gene that is directly and transcriptionally regulated by vascular MR. These data suggest that direct inhibition of vascular MR activity may play a role in the protective effects of MR antagonists in clinical trials.

Whole vessels, rather than cultured cells, were used for these expression studies because vascular cell function is known to be regulated by complex paracrine signaling between distinct cell types in the vasculature (40–42). Indeed, the enhanced regulation

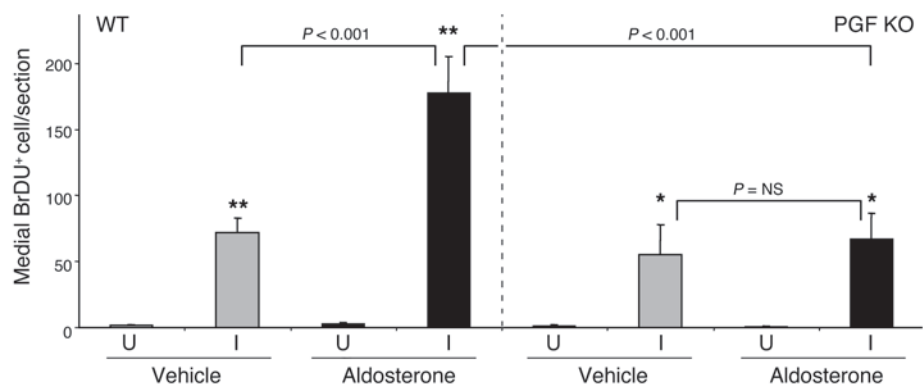
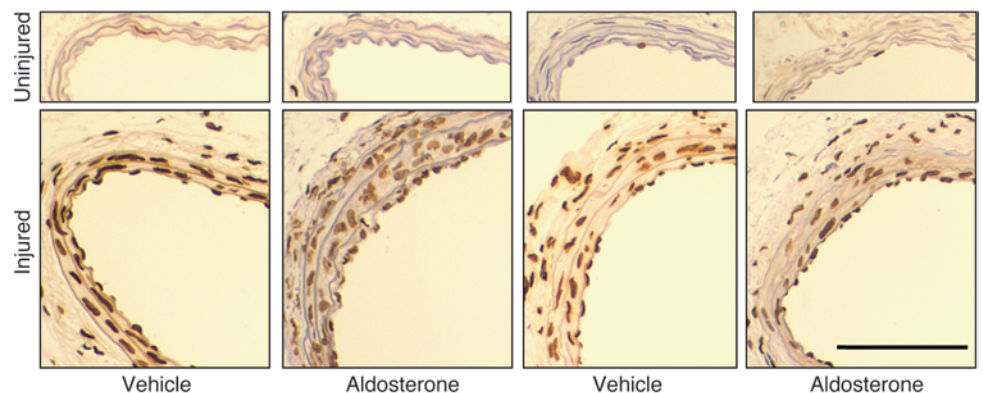


Figure 5

Aldosterone-enhanced SMC proliferation after vascular injury is inhibited in PGF-deficient mice. Medial SMC proliferation was quantified in BrdU-stained sections of uninjured (U) and injured (I) WT and PGF KO mouse carotid arteries treated with vehicle or aldosterone. Medial VSMC BrdU labeling data for all animals is indicated at top. Representative carotid artery sections are shown at bottom (scale bar: 1 mm). * $P < 0.01$, ** $P < 0.001$ versus uninjured.



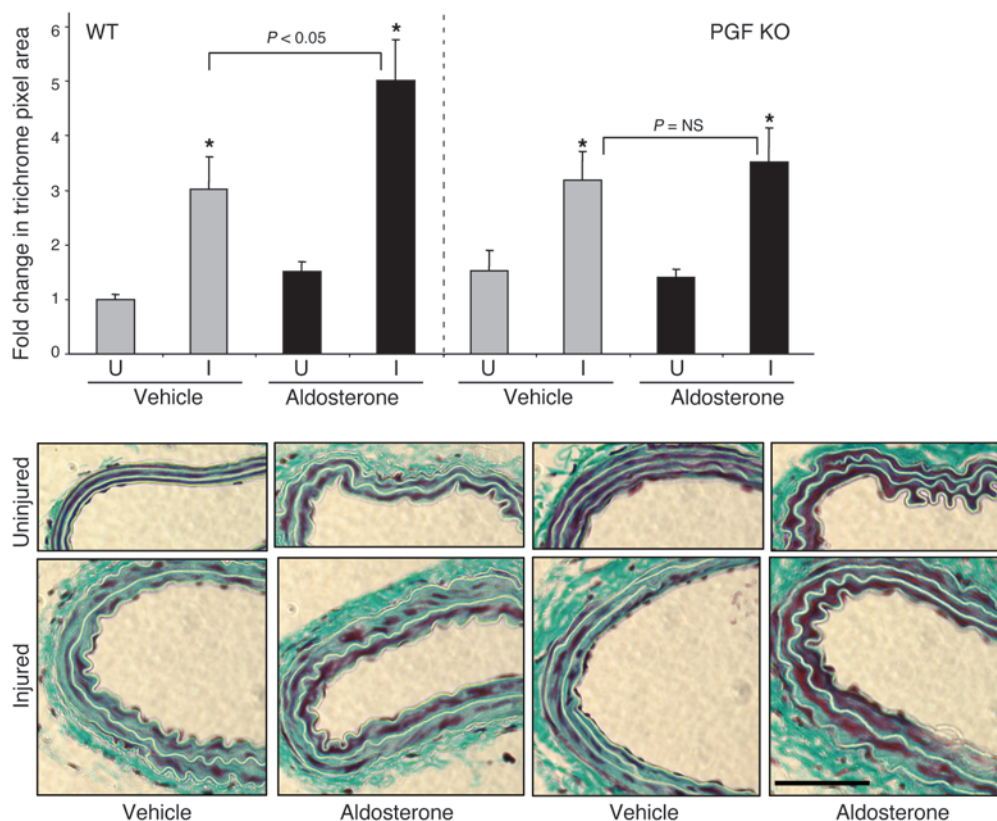


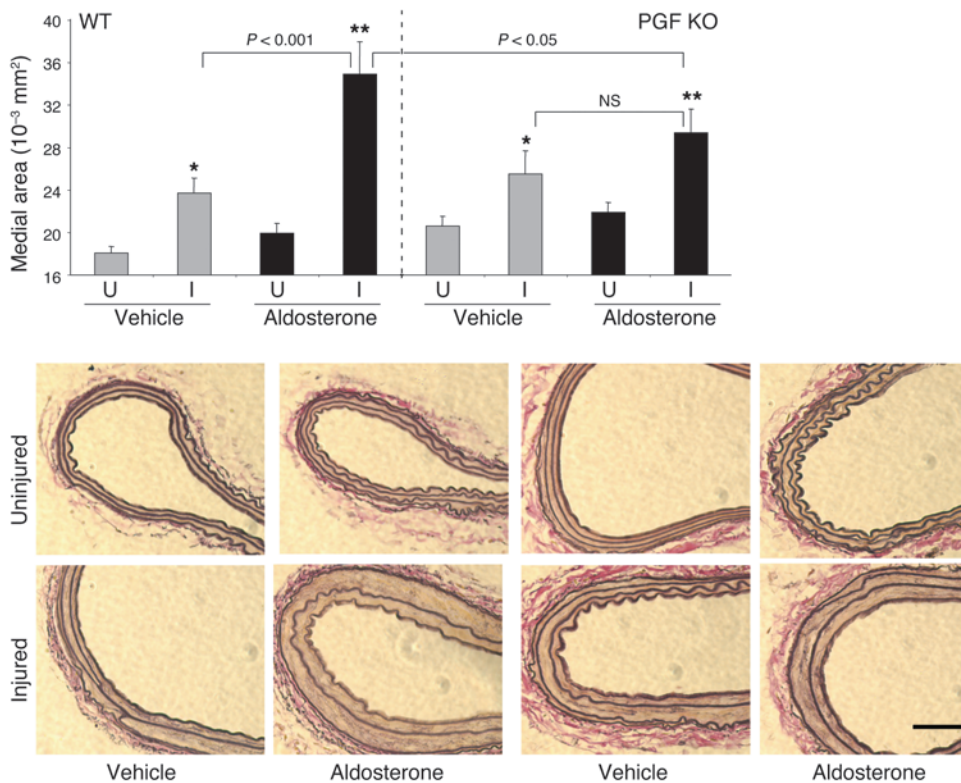
Figure 6 Lack of aldosterone-enhanced ECM deposition after vascular injury in PGF-deficient mice. ECM deposition was quantified in trichrome-stained sections of uninjured and injured WT and PGF KO mouse carotid arteries treated with vehicle or aldosterone. Fold change in medial trichrome pixel area for all animals compared with WT vehicle-treated, uninjured vessels is indicated at top. Representative carotid artery sections are shown at bottom (scale bar: 1 mM). * $P < 0.001$ versus uninjured.

of PGF by aldosterone in the absence of the endothelium supports the hypothesis that multiple cell types interact to determine the ultimate magnitude of the transcriptional responses to aldosterone in the vessel. Using this approach, it is difficult to precisely determine the cell type in which aldosterone regulation occurs. Overall, the data support the hypothesis that PGF and Flt1 are induced by aldosterone in VSMCs in injured vessels. Specifically, when the endothelium is denuded from vessels, PGF and Flt1 expression are enhanced by aldosterone (Figure 3), suggesting that this regulation does not require the endothelium. Since SMCs constitute the majority of the murine vessel under the denuded conditions, changes in mRNA abundance likely represent predominantly SMC effects. In addition, others have shown that in the presence of endothelial injury, Flt1 (which is expressed predominantly in ECs in healthy vessels) is expressed on SMCs (43). However, the contribution of vascular fibroblasts and/or inflammatory cells cannot be excluded.

Despite a significant increase in vascular Pgf secretion from aldosterone-treated vessels (Figures 1, 3, and 4), circulating serum Pgf levels did not increase in WT mice after aldosterone infusion. This may be due to a rapid but temporary upregulation of Pgf by aldosterone after vascular injury that could alter mechanisms driving SMC proliferation over 2 weeks, despite normal serum levels at the time of animal sacrifice. Alternatively, local aldosterone-stimulated Pgf release may induce paracrine effects on vascular and inflammatory cells in the environment of the injured vessel without significantly altering systemic Pgf levels. The latter mechanism is supported by the dramatic increase in aldosterone-stimulated Pgf secretion from carotid arteries that would be expected to result in high local Pgf levels in these small vessels without altering sys-

temic Pgf levels. This local paracrine mechanism is consistent with a role for Pgf in mediating aldosterone-induced remodeling at the site of vascular injury and a lack of effect of aldosterone elsewhere in the animal, where the vessel is intact.

Animal studies have shown that aldosterone, acting via MR, exacerbates vascular injury responses to a variety of conditions that cause endothelial dysfunction, including mechanical EC injury (13, 14, 16), uninephrectomy/high salt-induced hypertension (15), or hyperlipidemia-induced vascular damage (44). Our carotid injury model suggests that the detrimental vascular effects of aldosterone occur only with the concomitant presence of endothelial injury, but the ex vivo experiments demonstrate that aldosterone upregulates PGF expression even in normal vessels (Figure 2). However, the data in injured mouse vessels (Figure 3) and in diseased human vessels (Figure 4) demonstrating enhanced aldosterone-stimulated PGF and Flt1 expression in the setting of injury supports several hypotheses to explain this apparent paradox. One possibility would be a threshold effect in which PGF promotes medial thickening only when present at sufficiently high local concentrations, as in the environment of the injured vessel. Additionally, previous studies have demonstrated that in healthy vessels, Flt1 receptors are expressed exclusively on ECs, where they mediate vasodilation and EC survival in response to PGF while in the presence of vascular injury, Flt1 is expressed on SMCs, where it mediates vasoconstriction and SMC proliferation in response to PGF (43, 45, 46). These observations support the model that in uninjured vessels the modest aldosterone-stimulated PGF release acts only on ECs, while in injured vessels the Flt1-expressing SMCs respond to PGF by proliferating (43), with enhanced responsiveness following aldosterone treatment due to MR-mediated increases in the amount of PGF released and the amount of Flt1 on

**Figure 7**

Aldosterone-enhanced medial vessel thickening after vascular injury is inhibited in PGF deficient mice. Medial vessel area was quantified in Elastin-stained sections of uninjured and injured WT and PGF KO mouse carotid arteries treated with vehicle or aldosterone. Medial area data for all animals is indicated at top. Representative carotid artery sections are shown at bottom (scale bar: 1 mm). * $P < 0.05$, ** $P < 0.001$ versus uninjured.

the SMCs of the injured vessel. This model is consistent with the hypothesis that the endothelium inhibits aldosterone-stimulated PGF and Flt1 expression in healthy vessels, and that this protection may be lost in disease states that cause EC dysfunction such as atherosclerosis. The mechanism by which the endothelium attenuates aldosterone-stimulated PGF and Flt1 expression remains to be investigated. Taken together, these data support what we believe is a novel mechanism that explains the clinical finding that MR antagonists decrease cardiovascular mortality and ischemia in patients with underlying EC dysfunction despite virtually normal serum aldosterone levels, and that modest increases in serum aldosterone in patients with atherosclerosis promotes carotid medial thickening, cardiovascular ischemia, and death (2–4, 6, 7, 47).

Genetic deletion of other factors in mice, such as apoptosis signal-regulating kinase 1 (ASK1), growth arrest specific protein 6 (GAS6), and NADPH-oxidase 2 (Nox2), has been shown to modulate aldosterone-induced cardiovascular damage (48–51). In all of these prior studies, mice were infused with a high dose of aldosterone (or deoxycorticosterone, the major murine mineralocorticoid) and fed a high-salt diet and/or underwent uninephrectomy, which resulted in significant hypertension. The aldosterone-mediated endpoints that were modified by the gene knockouts in these studies included cardiac hypertrophy, perivascular and interstitial cardiac inflammation and fibrosis, and cardiac oxidative stress. The present study demonstrates a relationship specifically between a VEGF signaling pathway and aldosterone-stimulated augmentation of vascular damage. While factors other than PGF surely contribute to aldosterone-stimulated vascular injury, several aspects of the experimental models in this study support distinct mechanisms and an important role for PGF. First, the low-dose aldosterone infusion (without high-salt intake) used in these studies did not raise blood pressure,

thus suggesting a direct effect of aldosterone on MR in the injured vessels (rather than secondary blood pressure effects from renal MR activation). The endpoints of aldosterone-stimulated SMC proliferation and fibrosis in non-cardiac vessels also have not, to our knowledge, previously been studied at a mechanistic level in vivo. In addition, our in vitro studies support vascular MR-dependent and transcriptional mechanisms by which aldosterone directly regulates vascular expression of PGF that may be mediated by a novel MRE in the *PGF* gene. Also, the data in injured mouse vessels suggest that vascular injury enhances both PGF and Flt1 expression, providing a mechanism for synergism between aldosterone and other forms of vascular injury. Finally, we present data demonstrating that aldosterone regulates production of PGF and Flt1 in vessels from patients with atherosclerotic vascular disease, and that the clinically vascular protective MR antagonist spironolactone inhibits PGF expression in diseased human vessels, supporting the potential for this pathway to play a role in human vascular disease.

Several limitations and areas of further study are worth noting. First, the wire carotid injury model was chosen for these studies because it is a reproducible method for examining the in vivo mechanisms regulating medial VSMC proliferation and fibrosis. However, aldosterone also has been shown to contribute to neointima formation (13, 16) and atherogenesis (44), processes that will need to be explored in distinct animal models. Although the molecular mechanism of aldosterone's contribution to neointima formation remains unknown (13, 16), the pathways controlling SMC proliferation in the media and the intima likely overlap, and hence the MR/PGF/Flt1 pathway may also play a role in intimal SMC proliferation, although this remains to be formally tested. Neointima formation requires VSMC migration in addition to proliferation (13), and the role of PGF in this process will need to be



explored in distinct animal vascular injury models that are known to have a reproducible neointimal response. Aldosterone also promotes atherosclerosis in mouse hyperlipidemia models by enhancing inflammatory cell recruitment to early atherosclerotic lesions (44), and PGF is a pro-inflammatory factor that promotes macrophage chemotaxis. Thus the role of PGF in aldosterone-stimulated vascular inflammation and atherosclerosis merits further exploration as well. In addition, we show here that PGF-deficient mice have moderate and significant increases in systolic blood pressure compared with PGF-intact controls, a finding that has not, to our knowledge, been previously reported but is consistent with the known vasodilatory effect of PGF (46) and may independently alter vascular function. Increased blood pressure would be expected to exacerbate the aldosterone effect on vascular remodeling, and the significant decrease in aldosterone-stimulated SMC proliferation and vessel thickening in the PGF KO mice, despite increased blood pressure, further supports a critical role for PGF in these processes. The finding of elevated blood pressure in PGF KO mice also supports potential novel vascular mechanisms of hypertension with relevance to other conditions such as pre-eclampsia (pregnancy-associated hypertension with renal and endothelial dysfunction), in which soluble Flt1 levels are known to be dramatically elevated, resulting in sequestration of PGF (reviewed in ref. 52), and might be considered when evaluating PGF antagonist drugs that are currently in use or in development as anti-angiogenic agents (38). An inherent limitation of the human tissue studies is the difference in procurement methods of human tissues from transplant donors (hours after harvest in cold cardioplegia) compared with CABG patients (minutes after harvest in cold DMEM). This unavoidable shortcoming must be considered when interpreting data that compares samples from the two sources. However, this limitation does not detract from the finding that treatment of tissue from patients with atherosclerotic vascular disease with the clinically beneficial MR antagonist spironolactone suppresses PGF expression and release, supporting that this mechanism may be relevant in human vascular disease. Finally, although we find that aldosterone specifically regulates vascular expression of PGF and the Flt1 receptor, this does not exclude the possibility that other VEGF ligands and receptors are involved in aldosterone-mediated vascular injury. VEGFA and VEGFB also bind to and signal through Flt1 receptors, and enhanced Flt1 occupancy by PGF has been proposed as a mechanism of liberating VEGF from Flt1 and enhancing VEGF signaling through Flk1 receptors; furthermore, a role for PGF/VEGF heterodimers in vascular cell function has been proposed (53). This study provides a mechanistic link between the renin-angiotensin-aldosterone system and regulation of the VEGF family and supports further investigation into the role of other VEGF family members in aldosterone-mediated vascular diseases.

These data have potential implications for developing new therapeutic strategies for vascular disease. Current therapies that limit aldosterone-mediated vascular injury, including angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and aldosterone antagonists, are effective but have significant and often limiting side effects caused by inhibition of renal MR (hyperkalemia), off-target effects on non-mineralocorticoid steroid hormone receptors (gynecomastia), and aldosterone-independent alterations in renal filtration (renal failure) (1, 2, 47). The identification of PGF as a vascular MR-regulated gene involved in vascular remodeling suggests that inhibition of PGF and/or Flt1 receptor signaling may be effective and more specific targets to prevent vas-

cular cell proliferation and fibrosis after vascular injury. A variety of antagonists of this pathway have already been generated, including anti-PGF antibodies and PGF-squelching, soluble Flt1 derivatives; these are being tested and used in humans with angiogenic disorders, including age-related macular degeneration and cancer (38) and may be useful in other forms of ischemic vascular injury and endothelial dysfunction.

In conclusion, these data support that transcriptional regulation of PGF by vascular MR is a new and important mechanism of aldosterone-mediated vascular injury. The identification of PGF as a novel vascular MR target gene with enhanced expression in the setting of endothelial damage and atherosclerosis may explain the substantial vascular protective effects of aldosterone antagonists in cardiovascular patients and supports the potential for PGF and its signaling pathway as a novel drug target for ischemic cardiovascular disease.

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 were purchased from The Jackson Laboratory, FVB WT mice (the background strain for PGF KO mice) were purchased from Charles River Laboratories, and PGF KO mice were provided by P. Carmeliet (54). Male mice weighing 22–30 grams were used for carotid injury experiments after confirming by Evan's blue staining that the 0.014 French wire induced complete endothelial denudation of the carotid artery in animals of this size. For the carotid injury experiment shown in Figures 5–7, PGF^{-/-} mice were compared with PGF^{+/+} littermate controls or to PGF-intact FVB mice. As we found no difference in any baseline or post-infusion characteristics (blood pressure, body weight, serum aldosterone levels, or vascular injury responses) between the littermate controls and the FVB mice, and the FVB mice have been previously well validated as WT controls for the PGF KO model (22, 53–55), we pooled the data from these 2 groups and refer to them as “WT controls” throughout the manuscript.

Blood pressure measurement. Blood pressure was measured prior to carotid injury and again after 2 weeks of vehicle or aldosterone infusion by tail cuff plethysmography using the Kent coda system. Mice were trained twice daily with 20 tail cuff inflations for at least 3 days. The morning after training, blood pressure was measured with 20 cuff inflations, the values averaged, rare outliers (± 2 SD from mean) eliminated, and systolic blood pressure recorded as the mean of the remaining measurements (provided there were at least 5 remaining values). Blood pressure data analysis was performed after the termination of the experiment by an investigator blinded to the genotype and treatment of the animals.

Wire carotid injury model. The wire injury protocol has been previously described in detail (32). Briefly, 1 day prior to carotid injury, mice were anesthetized, and an osmotic minipump (Alzet) was inserted subcutaneously to infuse aldosterone (6 $\mu\text{g}/\text{kg}/\text{d}$; Sigma-Aldrich) (dissolved in 100% ethanol and diluted in saline, infused at 0.25 $\mu\text{l}/\text{h}$) or vehicle (11% ethanol in saline). One day after pump implantation, endothelial denudation vascular injury was produced by intraluminal introduction of a thin wire into the left common carotid artery, and a 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, serum was collected for aldosterone and PGF quantification (see below for methods), and both carotid arteries were perfusion-fixed, harvested, and embedded in paraffin. Carotid artery sections were obtained at the identical distance from the carotid bifurcation, and serial parallel sections were stained with Elastin stain, trichrome stain, smooth muscle-specific α -actin, and BrdU as described previously (32). Medial area and ECM were quantified using computerized morphometric analysis on elastin and trichrome-stained sections, respectively, and medial



BrdU-labeled SMCs were identified by comparing parallel carotid sections stained with BrdU and smooth muscle-specific α -actin and quantifying as previously described (32). 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 ECs. Between 12 and 16 animals were included in each group.

Ex vivo vessel treatment. Discarded, de-identified human aortic tissue was collected at the time of surgery with approval from the New England Organ Bank and the Tufts Medical Center Institutional Review Board. "Normal" human aorta was collected from heart transplant donors, and "atherosclerotic" human aorta was collected from aortic punches made at the time of CABG anastomosis. Mouse aortas and/or carotid arteries were harvested from male C57BL/6 mice 10–16 weeks old that had been pretreated with spironolactone (20 mg/kg/d) via subcutaneous pellet (Innovative Research of America) for 5 days to suppress basal MR-mediated gene transcription. Mouse and human vessels were treated ex vivo at 37°C in DMEM medium (Gibco) with aldosterone (Sigma-Aldrich; 100 nM for 8 hours unless otherwise indicated) in the presence or absence of spironolactone (Research Plus Inc.; 1 μ M for Figure 4D, 10 μ M for Figure 1 and Figure 2B), actinomycin D (Sigma-Aldrich; 5 μ g/ml, pretreatment 1 hour), or CXMD (Sigma-Aldrich; 40 μ g/ml, pretreatment 1 hour). For Figure 4, C and D, human atherosclerotic aortic punches were treated with vehicle or spironolactone (1 μ M) for 18 hours in DMEM supplemented with 10% bovine serum to mimic basal MR activation in vessels exposed to serum. Vessel tissue was then frozen in liquid nitrogen, RNA was isolated for use in microarray analysis and quantitative RT-PCR (QRT-PCR), and conditioned media were collected for growth factor quantification as described below. For all ex vivo experiments, aldosterone and spironolactone were dissolved in DMSO (Sigma-Aldrich), and appropriate vehicle controls were used for all experiments.

QRT-PCR. Total vessel RNA was isolated and reverse transcribed, and QRT-PCR was carried out as previously described (56) with primers specific for PGF, Flt1, or Flk1 (see Supplemental Table 2 for primer sequences). The CT value for each gene was normalized to that of β_2 -microglobulin (for mouse tissues) or GAPDH (for human tissues) and expressed as fold change in treated samples compared with normal or vehicle-treated samples as previously described (56). Between 3 and 6 mouse or human vessels were used for each condition.

Serum analysis. Mouse serum aldosterone levels were measured by COAT-A-COUNT Aldosterone Radioimmunoassay (Diagnostic Products Corporation), and PGF levels were measured by Quantikine mouse PGF2 ELISA (R&D Systems).

Vessel protein analysis. Whole mouse aortic protein lysate was made by crushing each vessel (from the aortic valve to the iliac bifurcation) in a glass homogenizer in 500 μ l of Lysis Buffer (50 mM Tris (tris[hydroxymethyl]aminomethane) pH 7.4, 150 mM NaCl, 0.02 M phenylmethylsulfonyl fluoride, 50 g/ml leupeptin, and 50 g/ml aprotinin). The lysate was then pressed 10 times through a 25-gauge syringe, incubated on ice for 30 minutes, and centrifuged for 10 minutes at 16,000 g at 4°C. Growth factor protein was measured in this aortic vessel supernatant and/or conditioned media from hormone-treated vessels using Quantikine ELISA kits: mouse PGF2, human PGF, and mouse VEGF (R&D Systems).

Transfection and reporter assays. The PGF MRE site was cloned between the Xho1 and Sac1 sites in PGL3-Basic vector (Promega) and confirmed by sequence analysis (mouse chromosome 12 base pairs 86543311–86543327, CAATGTCATGACAAGA). HEK293 cells grown in 10% charcoal-stripped bovine serum were transfected with the luciferase reporter vector and 100 ng of empty expression vector (CMX) or the same vector containing the human MR sequence (57). Eighteen hours after transfection, aldosterone or spironolactone was added, and 24 hours later the cells were lysed and luciferase activity measured as previously described (20). Transfection efficiency was normalized to β -galactosidase activity from a cotransfected plasmid (20). Three to five experiments were performed, each in duplicate.

Statistics. Values are reported as mean \pm 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.

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