Direct regulation of blood pressure by smooth muscle cell mineralocorticoid receptors

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Hypertension is a cardiovascular risk factor present in over two-thirds of people over age 60 in North America; elevated blood pressure correlates with increased risk of heart attack, stroke and progression to heart and kidney failure. Current therapies are insufficient to control blood pressure in almost half of these patients1,2. The mineralocorticoid receptor (MR), acting in the kidney, is known to regulate blood pressure through aldosterone binding and stimulation of sodium retention3. However, recent studies support the concept that the MR also has extrarenal actions4–7 and that defects in sodium handling alone do not fully explain the development of hypertension and associated cardiovascular mortality8,9. We and others have identified functional MR in human vascular smooth muscle cells (SMCs)10,11, suggesting that vascular MR might directly regulate blood pressure. Here we show that mice with SMC-specific deficiency of the MR have decreased blood pressure as they age without defects in renal sodium handling or vascular structure. Aged mice lacking MR in SMCs (SMC-MR) have reduced vascular myogenic tone, agonist-dependent contraction and expression and activity of L-type calcium channels. Moreover, SMC-MR contributes to angiotensin II–induced vascular oxidative stress, vascular contraction and hypertension. This study identifies a new role for vascular MR in blood pressure control and in vascular aging and supports the emerging hypothesis that vascular tone contributes directly to systemic blood pressure.

The renin-angiotensin-aldosterone system is a hormonal cascade with a well-recognized role in blood pressure regulation. Angiotensin II (AngII) acts via the angiotensin type 1 receptor (AT1R) on vascular cells to cause vasoconstriction and on adrenal cells to cause release of the hormone aldosterone that activates renal MR to modulate sodium balance12. Mice deficient in the MR in all tissues die in the neonatal period from salt wasting, which is consistent with the known role of the MR in regulating vascular volume13,14. However, mice with renal tubule–specific MR deficiency survive unless challenged with low-salt conditions15,16, supporting the concept that loss of extra-renal MR contributes to the hypertension and mortality associated with complete MR deficiency. MR antagonists prevent activation of the MR11,12 and decrease blood pressure and cardiovascular mortality17–19. A recent analysis of MR antagonist trials for the treatment of hypertension concluded that a component of the blood pressure–lowering effect of MR antagonism can be distinguished from effects on urinary electrolyte excretion, supporting the relevance of renal-independent regulation of blood pressure by the MR in humans6,20. Mice overexpressing the MR in endothelial cells have high blood pressure2, but the role of endogenous vascular MR in the regulation of blood pressure has not been explored.

We generated mice lacking MR specifically in SMCs by first creating mice with a loxP-flanked MR (MRfloxed) allele and then breeding these mice with mice containing the SMA-Cre–ERT2 transgene, in which the smooth muscle actin promoter drives expression of the tamoxifen-inducible Cre-ERT2 recombinase21 (Supplementary Fig. 1a). Comparisons were made between tamoxifen-treated MRfloxed/SMA-CreERT2–positive (Cre+) mice and tamoxifen-treated MRfloxed/SMA-CreERT2–negative (Cre−) littermate controls. After treatment of Cre+ mice with tamoxifen, the gene encoding MR (Nr3c2) remained intact in tissues lacking substantial SMC populations (Fig. 1a), but vascular SMCs showed near-complete excision of Nr3c2 and loss of MR mRNA expression (Fig. 1b,c and Supplementary Fig. 1); aortic mRNA expression of angiotensin receptors (AT1a, AT1b and AT2) was not altered (data not shown). In addition, we observed no alterations in feeding or growth in Cre+ mice compared to Cre− littermate controls (Supplementary Table 1 and data not shown).

Telemetric blood pressure monitoring revealed lower blood pressure in male Cre+ mice compared to Cre− littermates (Fig. 1d). This blood pressure difference increased with age, becoming significant by age 7 months. We assessed contraction and relaxation of mesenteric resistance arteries (MRAs) in adult (3–4 months old) and aged (>9 months old) mice. Vessels from Cre+ adult mice showed a modest increase in contraction to phenylephrine and enhanced endothelial-dependent vasodilatation compared to vessels from Cre− controls, consistent with the lack of a significant difference in the blood pressure

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of adult Cre− and Cre+ mice (Fig. 1e and Supplementary Fig. 2). MRAs from aged Cre− mice showed significantly augmented contraction in response to KCl or the thromboxane receptor agonist U46619 compared to MRAs from Cre− adult mice (Fig. 1e). This age-dependent increase in contraction response was lost in aged Cre+ littermates (Fig. 1e) and was associated with a modest decrease in endothelial cell–independent vasodilatation in MRAs from aged Cre+ compared to aged Cre− mice (Supplementary Fig. 2). Phenylephrine-induced contraction increased with age regardless of genotype, indicating that not all age-dependent increases in contractile responses are lost in this model (Fig. 1e). These data support a direct role for SMC-MR in blood pressure regulation by modulating the intrinsic vascular function of resistance vessels, particularly with aging.

As the MR contributes to blood pressure control by modulating renal sodium handling, we investigated the salt sensitivity of blood pressure in Cre+ and Cre− mice. In adult mice, there was no blood pressure difference between genotypes, regardless of dietary sodium intake (Fig. 2a). The lower blood pressure of aged Cre+ mice compared to aged Cre− mice was maintained under both high- and low-sodium conditions (Fig. 2b) and throughout the diurnal cycle (Supplementary Fig. 3). We observed no difference in serum or urine electrolytes or in serum aldosterone levels between Cre+ and Cre− mice (Supplementary Table 1). Treatment of mice by providing them with 1% NaCl drinking water together with infusion of aldosterone significantly increased blood pressure to similar levels in adult and aged mice, and these responses were independent of genotype (Fig. 2c,d).
Figure 3  Normal vascular structure with decreased tone in mice with SMC-specific MR deficiency. (a,b) Elastin (a) and trichrome (b) staining of representative thoracic aorta sections from 3-, 9- and 18-month-old Cre− and Cre+ litters. Medial area and collagen content are quantified on the right (n = 3 mice per group). (c) Passive vessel area, lumen area and distensibility in cannulated MRAs from aged (>9-month-old) Cre− and Cre+ mice over a range of intraluminal pressures (n = 7 mice per group). (d) Passive and active diameters of cannulated MRAs from aged Cre− and Cre+ mice over a range of intraluminal pressures. (n = 5–8 mice per group). P < 0.001 for active versus passive tone. Average spontaneous tone at 70 mm Hg is calculated as the percentage decrease in active lumen diameter from the passive diameter and compared in the graph on the right. (e) Mesenteric SMC patch-clamp studies. Top graphs show representative whole-cell K+ current recordings for Cre− and Cre+ mesenteric SMCs. The BKCa component is isolated by inhibition with the BKCa inhibitor iberiotoxin (IBTX; 10−7 M) and voltage-gated K+ channels (Kv) by inhibition with 4-aminopyridine (4-AP; 10−3 M). Middle graphs show group data; bottom graphs show responses to the BKCa activator NS1619, presented as percentage of basal current (n = 4 mice per group). (f) Expression of BKCaα (Kcnma1 gene), BKCaβ (Kcnmb1 gene), and Cav1.2 (Cacna1c gene) mRNA in aortas isolated from Cre− and Cre+ aged mice by quantitative RT-PCR (n = 5–7 mice per group). (g) Contractile responses of pressurized MRAs (at 70 mm Hg) to phenylephrine or L-type calcium channel activation with BayK8644, represented as the percentage decrease in MRA diameter upon agonist treatment of aged vessels (n = 5–8 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001. Values are reported as means ± s.e.m.

On a low-sodium diet, aged mice showed the expected decrease in urinary sodium excretion and fractional excretion of sodium, regardless of genotype (Fig. 2c,f). These results are consistent with intact MR function in the kidney tubules of these mice and support an extrarenal, SMC-MR–dependent mechanism for blood pressure regulation.

We next examined the effect of SMC-MR deficiency on vascular structure. Aortic medial area and collagen content increased with age independently of the presence of SMC-MR (Fig. 3a,b). In 18-month-old mice, cardiac hypertrophy occurred in Cre− but not Cre+ mice (Supplementary Fig. 4), consistent with the end-organ effects of longstanding exposure to lower blood pressure in the Cre+ mice. As resistance vessels are crucial in modulating blood pressure, we examined the structural characteristics of pressurized MRAs from aged mice. Vessels from aged Cre+ and Cre− mice had similar passive vessel and lumen diameters and similar values for area, stress, strain, distensibility and stiffness over a range of intraluminal pressures (Fig. 3d and Supplementary Table 1). However, MRAs from aged Cre+ mice developed significantly less spontaneous myogenic tone compared to those from Cre− mice (Fig. 3d). These data support the hypothesis that SMC-MR contributes to vascular tone and blood pressure regulation in aged mice independently of vascular structural changes.

Voltage-gated calcium (Ca) channel activation contributes to myogenic tone, whereas large-conductance Ca-activated potassium channels (BKCa) counter-regulate myogenic constriction22. As coronary artery BKCa expression is reduced in mice with cardiac-specific aldosterone synthase overexpression23, we first examined BKCa activity. Patch-clamp studies of mesenteric SMCs from aged Cre+ and Cre− mice revealed no differences in total potassium (K+) current, the proportion of K+ current from BKCa or voltage-activated K+ channels, or the response of BKCa to activation with the agonist NS1619 or inhibition with iberiotoxin (Fig. 3e). mRNA expression of the BKCa subunits BKCaα1 and BKCaβ1 was not different between aged Cre+ and Cre− vessels, but expression of the L-type Ca channel Cav1.2 was significantly lower in Cre+ vessels (Fig. 3f). In addition, the contractile response of MRAs to the L-type Ca channel agonist BayK8644 was significantly lower in vessels from aged Cre+ mice compared to those from aged Cre− mice (Fig. 3g). These data support the concept that...
regulation of L-type Ca channels by SMC-MR underlies age-associated alterations in myogenic tone, agonist-induced contraction and blood pressure.

Renin-angiotensin-aldoosterone system signaling is recognized to be enhanced in the aging vasculature, contributing to the vascular aging phenotype24–26. Given that we previously demonstrated direct AT1R-dependent activation of MR by AngII in human SMCs11, we examined the effects of in vivo AngII infusion on blood pressure responses in SMC-MR deficient mice. As expected27, AngII infusion produced a robust hypertensive response in Cre− mice that was significantly enhanced in aged compared to adult mice (Fig. 4a,b). In adult Cre+ mice, the maximal pressor response to AngII was reduced by 31%, correlating with a 44% reduction in maximal contraction of MRAs in response to AngII (Fig. 4c,d). In aged Cre+ mice, there was no significant AngII pressor response, correlating with a lack of significant contraction in response to AngII in MRAs from aged Cre+ mice (Fig. 4b,d).

The pressor response to AngII involves production of reactive oxygen species (ROS)28, as confirmed by inhibition of the AngII pressor response in adult and aged mice by treatment with the SOD-mimetic TEMPO (Supplementary Fig. 5). We quantified basal and AngII-stimulated vascular ROS production by whole-vessel dihydroethidium staining of carotid arteries. In adult mice, there was no difference in basal vascular ROS production between Cre− and Cre+ mice, but AngII-stimulated ROS production was attenuated in Cre+ compared to Cre− mice (Fig. 4e). These findings are consistent with a lack of difference in basal blood pressure in adult Cre− and Cre+ mice and with attenuation of the AngII pressor response in Cre+ mice. In aged mice, basal vascular ROS production was significantly decreased in vessels from Cre− compared to Cre+ mice, and ROS production in Cre+ vessels did not increase in response to AngII (Fig. 4f). These results also correlate with the decreased basal blood pressure and loss of AngII pressor and contractile responses in aged Cre+ mice. These data support the concept that SMC-MR contributes substantially to AngII-induced vascular oxidative stress, vascular constriction and blood pressure elevation, particularly in the aging vasculature, and show that the previously described bidirectional crosstalk between MR and AT1R signaling in SMCs11,29 has in vivo relevance.

Together, these studies demonstrate that SMC-MR modulates vascular contractile function and tone independently of changes in vascular structure or defects in renal sodium handling, contributing to the blood pressure response to AngII and the age-associated increase in blood pressure. These findings support the emerging hypothesis that direct regulation of vascular tone contributes to the regulation of systemic blood pressure30 and are consistent with clinical studies suggesting extrarenal mechanisms underlying the development of hypertension and associated cardiovascular diseases8,9. These studies also support the concept that effects on vascular MR contribute to the electrolyte-independent antihypertensive effects of AT1R- and MR-antagonist drugs6,20. Notably, modulation of renal vascular tone could alter renal function, as new treatments for hypertension are targeting renal vascular sympathetic tone with some success31. Thus, although renal MR function was not affected in mice with SMC-MR deficiency, regulation of renal vascular tone by SMC-MR could have secondary effects on sodium handling that could also affect blood pressure control.

Vessels from aged SMC-MR−deficient mice had profoundly reduced myogenic tone and contraction to the G protein–coupled receptor (GPCR) agonists thromboxane and AngII (but not to the GPCR agonist phenylephrine, which can act through divergent signaling mechanisms). Thromboxane receptor antagonism inhibits AngII-induced hypertension32, supporting the idea that there is an overlap between the AT1R and thromboxane receptor signaling pathways that involves SMC-MR. Both pathways modulate Ca channel activity22,33.
which was reduced in aged SMC-MR deficient mice. Angiotensin-mediated ROS production has been shown to regulate vascular Cav1.2 expression14, suggesting a potential mechanistic link between reduced vascular ROS and Cav1.2 expression in these mice. Further exploration of the role of SMC-MR in the regulation of vascular GPCR signaling, Ca channel expression and oxidative stress is warranted.

Aging-associated hypertension is common, with an incidence approaching 80% in individuals over 80 years of age1. Although there is potential for some adaptive benefit to the rise in blood pressure with age, this phenomenon contributes substantially to the incidence of heart attack, stroke, atrial fibrillation and kidney and heart failure in the aging population worldwide1,2. Deficiency in SMC-MR prevented many aspects of cardiovascular aging in our mouse model, including increased blood pressure, cardiac hypertrophy, vascular contraction, blood pressure responsiveness to AngII and oxidative stress, supporting a role of SMC-MR as a global regulator of vascular aging. Currently used MR antagonists have beneficial effects on blood pressure and mortality in people with cardiovascular disease, but aging. Currently used MR antagonists have beneficial effects on blood pressure and mortality in people with cardiovascular disease, but hyperkalemia mediated by renal MR inhibition is a major limitation to their clinical use. Further study of the diverse roles of the MR and the signaling pathways it regulates in the vasculature may lead to the development of new therapies for common cardiovascular disorders, particularly in the aging population.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.M., M.A.H., M.E.M. and I.Z.J. designed the experiments. A.M., P.W.P., S.B.B., M.A., M.J.Z. and A.M.D. obtained the data and analyzed it with advice from I.Z.J. D.M. and P.C. created the MRff and SMA-Cre-ERT2 mice. A.M. and I.Z.J. wrote the manuscript. All authors participated in discussion, contributed ideas and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Generation of inducible SMC-specific MR knockout mice. All mice were handled in accordance with US National Institutes of Health standards, and all procedures were approved by the Tufts Medical Center Institutional Animal Care and Use Committee. A genomic region encompassing exons 5 and 6 of the Nr3c2 (MR-encoding) gene, encoding the hinge region and the N-terminal part of the ligand binding domain, was flanked by loxP sites via homologous recombination in embryonic stem cells, and MR reporter mice were generated according to standard procedures35. The loxP sites were inserted on mouse chromosome 8 between base pairs 77,185,582 and 77,185,583 and between 77,187,725 and 77,187,726 (University of California–Santa Cruz coordinates).

SMC-MR knockout mice were generated by crossing MRf/f mice with SMA-Cre-ERT2 mice (smooth muscle actin promoter driving expression of Cre-ERT2 recombinase that is activated by tamoxifen) that we have previously described21. Mice were born at Mendelian frequencies. For all studies, male MRf/fSMA-Cre-ERT2–positive (Cre+) and MRf/fSMA-Cre-ERT2–negative (Cre−) littermates were injected intraperitoneally with 1 mg tamoxifen daily for 5 days at age 6–8 weeks, and all studies were performed at least 4 weeks after tamoxifen injection to allow for Nr3c2 excision and for MR degradation. SMA-Cre-ERT2 mice were also crossed with Rosadc reporter mice (Jackson Labs), injected with vehicle or tamoxifen as described above and killed 2 weeks after the last injection. Tissues were removed, fixed and embedded in paraffin and stained with X-gal as described11.

PCR analysis of Nr3c2 genomic DNA. DNA was extracted and PCR was performed by standard methods using a combination of three primers: 5’-CCACTTTGATCCGCAATAAGTTGTC-3’, 5’-CAATTCTGAATCTGCTCCGAGGAGAAGCTCAGCCGAAACCTGACTTC-3’. Quantitative RT-PCR. RNA was extracted and reverse transcribed, and quantitative RT-PCR was performed with gene-specific primers as previously described36. Ct values were normalized to β2-microglobulin (B2m), and mRNA levels in Cre+ samples were expressed as a percentage of those in Cre− samples. Specific primers for quantitative RT-PCR for MR (nuclear receptor subfamily 3, group C, member 2; Nr3c2), Care2 (corticosteroid receptor type 2; Cnr2), Kcnma1 (conductance calcium-activated channel, subfamily M, β1 cation channel subunit; Cacna1c), B2m (β2-microglobulin) and Cav1.2 (calcium channel, voltage-dependent, L type, α1C subunit; Cacna1c) and B2m are listed in Supplementary Table 2.

Blood pressure measurement by telemetry. All blood pressure studies were performed using implantable blood pressure transmitters (Data Sciences International, TA11PA-C10) with n = 4–8 mice per group. Blood pressure was recorded for 60 s every 30 min as previously described37. Mice were maintained on a 12-h light–dark cycle, with normal chow (0.3% NaCl, Harlan diet TD8604) and water available ad libitum. For salt challenges, mice with telemetry devices were fed a low-salt diet (0.02% NaCl; Harlan diet TD90228) or a high-salt diet (6% NaCl; Harlan diet TD90230) for 5 d, and blood pressure on days 3–5 were averaged. For aldosterone and salt administration, osmotic minipumps were implanted (Alzet) to infuse AngII administration, osmotic minipumps were implanted (Alzet) to infuse AngII, PE and sodium nitroprusside (SNP) were built. For relaxation studies, vessels were preconstricted with U46619 at EC50 before administration of Ach and SNP (average preconstricted force = 6.6 mN Cre− adult, 7.2 mN Cre+ aged, 6.9 mN Cre+ adult, 7.2 mN Cre+ aged, P = not significant).

Mesenteric vessel structural and reactivity studies. For structure and distensibility studies, MRAs from n = 5–8 mice for each genotype were cannulated in a pressure myograph (Living System Instrumentation) and incubated in calcium-free PSS containing 2 mM EGTA and 1 μM SNP for analysis of passive structure over a range of intraluminal pressures (0 to 180 mm Hg) as described previously38. The elastic modulus (β-coefficient) was calculated from the stress strain curves for the individual vessels, and these curves were fitted to an exponential model (y = aeβx), where β is the slope of the curve: the higher the β-coefficient the stiffer the vessel. Distensibility was calculated as the percentage change in lumen diameter (LD) at a given intraluminal pressure (LDp) from LD at 3 mm Hg (LD0): [(LDp − LD0) / LD0] × 100 as described39. Myogenic reactivity was measured over a pressure range of 10–120 mm Hg in Ca2+–containing PSS. After active tone measurements, vessels were superfused with buffer lacking added Ca2+ and containing 2 mM EGTA. Passive-diameter responses were then recorded over the pressure range 10–120 mm Hg. Tone was calculated as the percentage decrease in LD from the passive LD at 70 mm Hg; % tone = [1 − (active diameter / passive diameter)] × 100 as described39. Contraction in response to BayK8644 (10−7 M) and PE (10−6 M) was assessed by video microscopy in pressurized (70 mm Hg) MRAs.

Whole-cell K+ channel recordings. Mesenteric artery SMCs were isolated as previously described40. K+ currents were measured using standard whole-cell recording techniques42. To obtain current–voltage relationships, cells were set at a holding potential of ~70 mV and voltage steps applied from ~70 to ~70 mV at 300-ms intervals. Components of the total K+ current were pharmacologically isolated using ibotenic acid (BKCa inhibitor; IBTX, 10−7 M, Sigma), 4-aminoypyridine (voltage-gated K+ channels inhibitor; 4-AP, 10−3 M) and NS1619 (BKCa agonist, 10−7 M, Sigma).

Dihydroethidium staining. Superoxide accumulation in carotid arteries was measured using dihydroethidium (DHE) staining. Vessels were incubated with vehicle (saline) or 200 nM AngII for 30 min (37 °C), rinsed and treated with 2 × 10−6 M DHE (Molecular Probes) for 45 min (37 °C in the dark). Vessels were washed and mounted on slides with ProLong Gold reagent (Invitrogen), and images were obtained over the length of the vessel using a fluorescent microscope (Nikon Optiphot-2) and SPOT advanced software. The mean fluorescence intensity for each vessel was determined by a blinded investiga tor using ImageJ software.

Statistical analyses. Within-group differences were assessed with two-factor or three-factor analysis of variance (ANOVA) or repeated-measures ANOVA (mesenteric vessel contraction studies) with Student-Newman-Keuls post-test. P < 0.05 was considered significant.

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