Aldosterone deficiency and mineralocorticoid receptor antagonism prevent angiotensin II-induced cardiac, renal, and vascular injury

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Abstract

Angiotensin II causes cardiovascular injury in part by aldosterone-induced mineralocorticoid receptor activation, and it can also activate the mineralocorticoid receptor in the absence of aldosterone *in vitro*. Here we tested whether endogenous aldosterone contributes to angiotensin II/salt-induced cardiac, vascular, and renal injury by the mineralocorticoid receptor. Aldosterone synthase knockout mice and wild type littermates were treated with angiotensin II or vehicle plus the mineralocorticoid receptor antagonist spironolactone or regular diet while drinking 0.9- saline. Angiotensin II/salt caused hypertension in both the knockout and wild type mice; an effect significantly blunted in the knockout mice. Either genetic aldosterone deficiency or mineralocorticoid receptor antagonism reduced cardiac hypertrophy, aortic remodeling, and albuminuria, as well as cardiac, aortic, and renal plasminogen activator inhibitor-1 mRNA expression during angiotensin II treatment. Mineralocorticoid receptor antagonism reduced angiotensin II/salt-induced glomerular hypertrophy, but aldosterone deficiency did not. Combined mineralocorticoid receptor antagonism and aldosterone deficiency reduced blood urea nitrogen and restored nephrin immunoreactivity. Angiotensin II/salt also promoted glomerular injury through the mineralocorticoid receptor in the absence of aldosterone. Thus, mineralocorticoid antagonism may have protective effects in the kidney beyond aldosterone synthase inhibition.

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INTRODUCTION

Interruption of angiotensin II (Ang II) signaling is an established strategy to reduce cardiovascular and renal events in high-risk populations.[1,2] Clinical trials also demonstrate that the addition of mineralocorticoid receptor (MR) antagonism to angiotensin-converting enzyme (ACE) inhibition reduces mortality and cardiac and renal injury,[3-5] suggesting that aldosterone contributes to Ang II-mediated injury or alternatively that MR mechanisms may in part be Ang- independent. In this regard, the glucocorticoids corticosterone or cortisol also bind to and activate the MR, raising the possibility that MR antagonism exerts beneficial effects that are independent of aldosterone or angiotensin. The observation that MR antagonism reduces end- organ damage even when endogenous aldosterone concentrations are suppressed further supports such a possibility.[6-8]

Pharmacologic aldosterone synthase (*As*) inhibition with FAD-286 reduces Ang II- induced profibrotic gene expression and cardiac and renal injury in rodent models of hypertension.[9,10] Because low levels of aldosterone remain in the circulation during treatment with FAD-286, it is not possible to exclude a permissive effect of aldosterone on Ang II signaling and tissue injury. *In vitro* studies also suggest that Ang II can activate the MR in the absence of aldosterone.[11,12] To investigate this *in vivo*, adrenalectomy eliminates circulating aldosterone non-selectively, requiring glucocorticoid replacement which may not mimic endogenous production. The availability of *As*-deficient mice (*As*^{-/-}) results in complete aldosterone deficiency without impaired glucocorticoid synthesis and permits investigators to dissect out any contribution of aldosterone to Ang II-mediated injury.[13,14]

We have previously used this model to determine the contribution of endogenous aldosterone to the acute effects of Ang II on inflammatory gene expression.[13] The current study tests the hypothesis that endogenous aldosterone contributes to chronic Ang II-induced pro- inflammatory gene expression and target organ damage via the MR.

RESULTS

Basic Metabolic Measurements

Aldosterone deficient mice (*As*^{-/-}) and wild-type (WT) littermates were treated with Ang II (1μg/hr) or vehicle via osmotic minipump plus the MR antagonist spironolactone (6mg/kg/day) or regular chow for 8 weeks while drinking 0.9- saline. Total body weight and lean body mass were similar among the treatment groups. Serum potassium was similar in the untreated *As*^{-/-} mice compared to WT (5.4±0.6 vs. 5.1±0.3, *P*=0.07), and among the treatment groups (**Table 1**). Serum sodium, chloride, bicarbonate, and corticosterone were also similar among the groups. Plasma aldosterone increased during Ang II treatment in WT mice and to an even greater extent during SPL treatment. Aldosterone was below the detectable limits in all *As*^{-/-} mice (*P*<0.01 vs. each WT group).

Urinary sodium excretion increased during Ang II treatment (**Table 1**). This response was blunted in *As*^{-/-} during both normal chow and SPL treatment. Urinary potassium excretion was not different among groups, except for an increase in the *As*^{-/-}-Ang II-SPL treated mice. Changes in urinary Na/K excretion paralleled those of urinary sodium excretion.

Blood Pressure Response to Ang II

Systolic blood pressure (SBP) was similar at baseline (101.9 ± 1.8 and 97.1 ± 2.3mmHg in WT and $As^{-/-}$, respectively; $P=0.13$). Vehicle treatment did not affect SBP (**Figure 1**). Ang II significantly increased SBP in both WT and $As^{-/-}$ mice ($P<0.05$). The pressor response to Ang II at *post hoc* testing was significantly decreased in $As^{-/-}$ mice in the presence ($P<0.05$) or absence ($P<0.05$) of SPL. SBP was similar in SPL-treated $As^{-/-}$ and WT mice ($P=0.20$).

Cardiac Assessment

Ang II treatment significantly increased cardiac mass in WT mice, but not in $As^{-/-}$ mice (**Figure 2A**). SPL prevented the effect of Ang II on cardiac mass in WT mice. Results were similar when correcting heart weight by either body weight (not shown) or tibia length. Ang II significantly increased cardiac interstitial fibrosis in WT, and this was prevented in $As^{-/-}$ mice (**Figure 2B**). Ang II did not significantly increase interstitial fibrosis in SPL-treated WT mice. Cardiac perivascular fibrosis was markedly increased by Ang II treatment in WT mice. The effect of Ang II was not significant in SPL-treated WT mice and was almost entirely abrogated in $As^{-/-}$ mice (**Figures 2C, 2D**).

Ang II increased aortic intima-media and adventitial thickness in WT (**Figure 3A, 3B, & 3C**; $P=0.002$ for Ang II effect, $P=0.05$ for SPL*Ang II effect on adventitial thickening). Either SPL or aldosterone deficiency attenuated aortic pathologic changes. There was no additive effect of SPL in aldosterone-deficient mice.

Kidney Injury Assessment

Blood urea nitrogen (BUN) increased significantly during Ang II treatment (**Figure 4A**). Neither SPL nor aldosterone deficiency prevented the Ang II-induced rise in BUN; however, combined aldosterone deficiency and SPL significantly prevented this increase. Ang II increased urine albumin excretion in WT mice, and this effect was abrogated by SPL treatment (**Figure 4B**) or aldosterone deficiency. Urinary albumin excretion was reduced during SPL treatment in Ang II-treated $As^{-/-}$ mice, although this effect was not significant ($P=0.07$ for $As^{-/-}$-Ang II vs. $As^{-/-}$-Ang II-SPL group).

Ang II increased average glomerular diameter ($P=0.003$); this effect was prevented by SPL ($P=0.01$ for Ang II*SPL interaction) but not by aldosterone deficiency (**Figure 4C**). Ang II treatment produced significant renal injury, as evidenced by tubular atrophy, perivascular and interstitial fibrosis, proteinaceous intratubular casts, and occasional glomerulosclerosis (**Figure 5A**). There was no effect of treatment on mesangial hypercellularity, mesangial expansion, or infiltrating inflammatory cells. Ang II treatment reduced nephrin immunoreactivity similarly in WT and $As^{-/-}$ mice (from 47.5- to 31.7- in Vehicle vs. Ang II treatment); SPL restored nephrin immunoreactivity WT and $As^{-/-}$ mice during Ang II treatment (42.2%; **Figure 5B**). Ang II treatment increased renal arterial media area ($P=0.04$); this was also attenuated by SPL ($P=0.02$), but not aldosterone deficiency (**Supplementary Figure 1A**). The increase in adventitial area in response to Ang II was prevented by SPL treatment ($P=0.03$), but was not affected by aldosterone deficiency (**Supplementary Figure 1B**). In contrast, there was no effect of either SPL or aldosterone

deficiency on Ang II-induced renal fibrosis or global kidney injury score (**Supplementary Figures 1C & 1D**).

Profibrotic Gene Expression

Ang II significantly increased mRNA expression of plasminogen activator inhibitor-1 (*Pai 1*) within cardiac, renal, and aortic tissue (**Figure 6**). Either SPL or aldosterone deficiency prevented the increase of *Pai 1* mRNA expression in these tissues to varying degrees. Cardiac prepro-endothelin-1 (*Et 1*) expression was unchanged (**Supplementary Figures 2A-C**). Ang II increased *Et-1* expression within renal tissue, and this effect was prevented by SPL but not by aldosterone deficiency. In contrast, aldosterone deficiency was associated with increased *Et-1* expression in the aorta.

Renal 11βhsd Gene Expression

We analyzed renal mRNA expression of 11-betahydroxysteroid dehydrogenase (*11βhsd)-1* and *11βhsd-2* gene expression during AngII administration. We observed no significant effect of Ang II on either *11βhsd-1* (1.12±0.2 fold change vs. Vehicle control; *P*=0.84) or *11βhsd-2* (1.18±0.2; *P*=1.0, n=5 for each group)

DISCUSSION

We found that endogenous aldosterone contributes to Ang II/salt-induced cardiac hypertrophy, cardiac fibrosis and aortic remodeling through an MR-dependent mechanism. In contrast, in the kidney Ang II/salt can also induce glomerular injury and arteriolar hypertrophy via activation of the MR, even in the absence of endogenous aldosterone. Ang II/salt causes renal interstitial fibrosis through an MR- and aldosterone-independent mechanism.

Numerous studies have demonstrated that aldosterone administration causes cardiac and aortic inflammation and remodeling, whereas MR antagonism prevents inflammation and fibrosis in salt-treated rodents.[15-20] Because endogenous aldosterone concentrations are suppressed during high-salt intake and ligands other than aldosterone can activate the MR, the absolute requirement for endogenous aldosterone in MR-mediated pathology has been called into question. Compelling studies in rats during excessive sodium intake suggest an aldosterone-independent, MR-dependent mechanism of cardiovascular and renal injury. For example, MR antagonism reduces cardiac hypertrophy in the Dahl salt-sensitive rat, a low-aldosterone model of hypertension, and the authors speculated that activation of the MR by glucocorticoids contributes to this effect.[6] In the heart, glucocorticoids may normally function as an MR antagonists under normal conditions, but contribute to MR activation depending on the redox state of the cell.[21] The MR also contributes to diastolic dysfunction and oxidative stress during low-dose Ang II administration, while aldosterone concentrations were unchanged.[22] In contrast, we observed that either MR antagonism or genetic aldosterone deficiency attenuated cardiac hypertrophy and cardiac perivascular injury, demonstrating a pivotal role for endogenous aldosterone in this model. These data are consistent with other studies testing the effect of pharmacologic aldosterone synthase

inhibition, and suggest that endogenous aldosterone contributes substantially to Ang II-induced cardiac injury.[9,10,23]

We have reported previously that spironolactone or pharmacologic aldosterone synthase inhibition with FAD286 ameliorated Ang II/salt-induced cardiac hypertrophy and interstitial fibrosis, but not coronary perivascular fibrosis in a rat model.[10] In the present study, genetic aldosterone deficiency or spironolactone prevented perivascular fibrosis as well as interstitial fibrosis and hypertrophy, suggesting that the lack of effect in the prior study may have been due to residual aldosterone production during pharmacologic inhibition. The modest and variable effect of spironolactone on interstitial and perivascular fibrosis in the heart may also reflect the use of low-dose spironolactone. The beneficial effect of high doses of spironolactone on these measures is well established.[24]

We have also demonstrated previously that either FAD-286 or spironolactone prevents AngII-induced BUN elevation, glomerulosclerosis, tubulointerstitial fibrosis, and perivascular remodeling in a rat model. In the current study, although Ang II caused significant renal injury, glomerulosclerosis was rare, and overall renal injury was not severe, likely reflecting a species and strain difference. Nevertheless, MR antagonism had selective beneficial effects on glomerular injury, beyond the effect of aldosterone deficiency, suggesting that activation of the MR by non-aldosterone ligands can contribute to injury.

That MR antagonism decreases Ang II-induced renal injury has been established previously. In humans with diabetic nephropathy, for example, MR antagonism reduces proteinuria during maximal ACE inhibition and may be more effective than additional renin-angiotensin-aldosterone system blockade with an Ang II receptor antagonist.[3] In rats treated with Ang II and a nitric oxide synthase inhibitor, adrenalectomy or MR antagonism with eplerenone prevents proteinuria and renal arteriolar and glomerular injury.[25] MR antagonism also protects against glomerular injury in salt-sensitive rats in which plasma aldosterone is suppressed, primarily via preserved podocyte function.[7,8,26]

To our knowledge, however, no studies have demonstrated pathologic effects of Ang II *in vivo* via MR activation, while excluding the possibility of endogenous aldosterone synthesis. Factors other than aldosterone can activate the MR in mesangial cells and podocytes, contributing to proteinuria and mesangial expansion. For instance, Rac1 GTPase contributes to podocyte injury via aldosterone-independent MR activation, producing proteinuria and renal injury.[27] Conditions such as hyperglycemia, obesity, and salt loading may activate the Rac1-MR pathway and contribute to injury.[27,28] The classical MR or a novel membrane-bound MR may be responsible for this aldosterone-independent signaling.[29] Our data suggest that other factors also act via the MR to produce glomerular expansion. Glucocorticoids can bind to and activate the MR. Normally glucocorticoids are inactivated by 11-betahydroxysteroid dehydrogenase (11βHSD)-2 in aldosterone target cells.[21] Rafiq *et al.* recently reported, however, that hydrocortisone causes renal injury in adrenalectomized rats through an MR-dependent mechanism, which could result from partial agonism of the MR in the absence of endogenous aldosterone or from stimulation of the MR on non-epithelial cells.[30] There was no significant difference in corticosterone concentrations to explain our findings. Alternatively, metabolic products of 11βHSD-2 could act as

physiologic MR antagonists or alter the redox state of the cell.[21,31] Glomerular *11βhsd-2* mRNA expression has been described in mesangial cells and podocytes.[32,33] We found no effect of Ang II on either *11βhsd-1* or *11βhsd-2* gene expression in the kidney, although we did not exclude an effect on 11βHSD-2 activity. Other investigators have also demonstrated that Ang II activates the MR via reactive oxygen species or NF-κB, providing another possible explanation for these findings.[22,34]

In contrast to the protective effect of MR antagonism on Ang II-induced glomerular expansion and nephrin immunoreactivity, neither MR antagonism with spironolactone or aldosterone deficiency prevented Ang II/salt-induced renal interstitial fibrosis in the current study. These findings are consistent with an AT_1 receptor-dependent profibrotic effect of Ang II, as other investigators have demonstrated.[35] The data are also consistent with the finding that spironolactone decreases glomerular injury, but not interstitial fibrosis.[8]

Multiple studies have demonstrated the central importance of PAI-1 as a profibrotic mediator in renal, vascular, and cardiac tissues.[36] Although Ang II is a classic stimulus for *Pai-1* gene expression, aldosterone and the MR are essential for a maximal response in certain tissues. We reported previously that endogenous aldosterone contributes to the effect of acute Ang II infusion on *Pai-1* mRNA expression within the heart, but not in aorta.[13] During chronic Ang II exposure, endogenous aldosterone appears to contribute to increased *Pai-1* expression within both the heart and the aorta via the MR. The concordant pathologic effects suggest a central role for aldosterone, the MR, and PAI-1, and suggest that improvement in our model could be accomplished via reduction of *Pai-1* expression.

Finally, genetic aldosterone deficiency resulted in a blunted pressor response to Ang II; decreased blood pressure may have contributed to the protective effect of aldosterone deficiency on cardiovascular remodeling. This underscores the lack of protective effect of aldosterone deficiency on glomerular expansion. Likewise, the pressor response to Ang II was similar in $As^{-/-}$-Ang II and WT-SPL-Ang II groups, suggesting that differences between the effects of MR antagonism and aldosterone deficiency on renal injury were blood pressure-independent.

Renin-angiotensin-aldosterone system blockade with either ACE inhibitors or Ang II receptor antagonists prevents cardiovascular and renal events in high-risk individuals, and spironolactone provides additional benefit beyond ACE inhibition.[3-5] Although ACE inhibition reduces circulating Ang II levels, other enzymes such as chymase contribute to local Ang II generation via an alternative pathway.[37] Although it is well established that aldosterone produces MR-mediated injury, other Ang II-induced ligands may also active the MR and contribute to tissue injury. The present study demonstrates that aldosterone mediates a portion of Ang II- induced cardiac, renal, and vascular injury and profibrotic gene expression. The additional benefit of MR antagonism in the setting of aldosterone deficiency suggests that Ang II can also mediate MR-dependent effects *in vivo* in the absence of aldosterone. Therefore MR antagonism may provide additional beneficial effects regardless of circulating aldosterone concentrations.

METHODS

Animals

All experiments were reviewed and approved by the Vanderbilt University Institutional Animal Care and Use Committee. *As$^{-/-}$* mice were generated on a 129 background[38] and backcrossed over 7 generations onto the C57Bl6/J strain (Jackson Laboratory, Bar Harbor, ME). Mice were genotyped by real-time PCR (Applied Biosystems 7900HT, Foster City, CA) using Taq-man probes for a sequence in the *Cyp11b2* gene and for a portion of the gene contained in the neomycin-resistance cassette as previously described.[13] Male *As$^{-/-}$* and wild type (WT) littermates were studied. Animals were housed in a temperature-controlled facility with a 12-hour light/dark cycle. Mice were maintained on 0.9- saline in the drinking water *ad libitum* starting at age 8 weeks and continued for the duration of the study.

Chronic Angiotensin II Infusion Protocol

At 12 weeks of age, mice were randomized to treatment with Ang II, spironolactone (SPL), or a combination of both. Ang II (1 μg/hr, CalBiochem, La Jolla, CA) or vehicle were delivered by osmotic minipumps (Alzet Model 2001, Alza Corp, Palo Alto, CA) implanted under pentobarbital anesthesia (50mg/kg IP) at 12 weeks of age. This dose of Ang II was chosen to induce modest hypertension and injury in WT mice.[39] After pump implantation, mice received either standard mouse chow (Purina Laboratory Rodent 5001) or standard chow supplemented with SPL, continued for the duration of the study. SPL chow was formulated by TestDiet (Richmond, IN) at concentrations to provide 6 mg/kg/day based on prior studies demonstrating that this dose decreases end-organ damage.[10,40] Minipumps were replaced after 4 weeks.

Mice were sacrificed 8 weeks after initial minipump implantation (20 weeks of age). Cervical dislocation was performed, the left renal artery was clamped, and blood was collected by cardiac puncture and transferred into a tube containing dipotassium-EDTA tubes (Microvette CB K2E, Sarstedt AG & Co). The base of the heart, the first 2 mm of descending aorta, and coronal sections of the kidney were fixed in 4% buffered paraformaldehyde overnight, transferred to 70% ethanol, and then embedded in paraffin for histological evaluation. The remainder of the heart, liver, and kidney were snap-frozen in liquid nitrogen for mRNA extraction. The remaining aorta (descending aorta to above the renal arteries) was transferred into fresh PBS solution, stripped of adventitial tissue, collected into RNA Later solution at 4° C overnight, and then transferred to a vial for storage at -80° C.

Cardiovascular measurements

Blood pressure was measured every 2 weeks starting at 6-8 weeks of age (4 weeks prior to randomization) using automated tail-cuff impedance plethysmography (BP-2000 Blood Pressure Analysis System; Visitech Systems, Apex, NC) in unanesthetized, trained mice, pre- warmed for 5 minutes at 37° C. At 20 weeks of age, transthoracic echocardiography was performed in conscious mice using a 15-MHz transducer (Sonos 5500 system, Agilent, Andover, MA) as previously published.[41]

Blood and Urine Chemistry

Blood was obtained for chemistry after 8 weeks of treatment, prior to sacrifice. Mice were fasted in a clean cage at 0900 hours, and 50μL whole blood was collected at 1300-1500 hours via saphenous vein into a heparinized capillary tube (Microvette CB K2E, Sarstedt AG & Co., Numbrecht, Germany) and processed immediately by iSTAT EC8+ cartridge (Heska Corp, Loveland, CO). An additional 30μL of blood was collected into dipotassium-EDTA (Microvette CB K2E, Sarstedt AG & Co) and centrifuged at 6,000 rpm for 5 minutes. Plasma was stored at -80° C until assay.

Aldosterone was determined using a radioimmunoassay utilizing ^{125}I-aldosterone (MP Biomedicals, Irvine CA), a primary antibody to aldosterone (NIDDK National Hormone & Peptide Program, Torrance CA), and a secondary anti-rabbit gamma globulin antibody (Linco Research, St. Charles, MO). Corticosterone was measured using a commercially available radioimmunoassay kit (ImmuChem Double Antibody Corticosterone Kit, MP Biomedicals, Irvine, CA).

For urine collection, mice were housed in individual metabolic cages (Nalgene® Labware, Rochester, NY) for 24 hours. Urine was collected while mice were ingesting normal chow and 0.9% saline *ad libitum* during the final week of treatment (i.e. Ang II, SPL). Urine Na^+ and Cl^- were measured by flame photometry (ILS 940). Urine was analyzed for albumin using an immunoturbidimetric assay and for creatinine by a modified colorimetric assay that correlates well with HPLC-based methods (DCA 2000®+ Analyzer and Microalbumin/ Creatinine cartridges, Bayer Corp, Elkhart, IN).[42]

Histopathologic Analysis

Images were captured on a Zeiss AxioScop 40 using MRGrab 1.0 (Media Cybernetics, Silver Spring, MD). All quantitative image analysis was performed using ImageJ version 1.40g (NIH, Bethesda, MD),[43] and all image measurements were calibrated using a 2mm slide micrometer photographed under appropriate magnification.

Glomerular area and diameter were determined by outlining 30-50 consecutive glomeruli per animal (imaged using 40x objective), for 3-4 animals per group. Renal media and perivascular area were measured by tracing the inner lumen, outer border of the tunica media, and outer border of the adventitial area (50× objective, 3-5 interlobular arteries per animal). Collagen area was quantified by picro-sirius red staining imaged under cross-polarized light, measured as percentage of collagen-positive area, excluding hilar vessels from the analysis. Aortic intima-media and adventitia thickness are averages from 4 linear measurements per aorta.

An investigator (A.B.F.) blinded to treatment assignment also assessed histopathology. Kidney injury was scored semi-quantitatively on masson trichrome-stained sections, from 0 to 3 for each high-power field (0 indicating no injury; 1 indicating single small focus of fibrosis; 2 indicating several small foci; 3 indicating multifocal fibrosis). Cardiac interstitial fibrosis was scored semiquantitatively as an estimate of percentage of fibrotic area. Cardiac perivascular fibrosis was scored semi-quantitatively (0 indicating no fibrosis; 1 indicating

adventitial fibrosis less than media area; 2 indicating fibrosis equal to media area; and 3 indicating fibrosis greater than media area).

Nephrin immunohistochemistry was performed after citrate buffer antigen retrieval, using an anti-mouse nephrin primary antibody (R&D Systems AF3159, Minneapolis, MN), biotinylated secondary antibody (Vector labs BA-5000, Burlingame, CA) and detection with the VECTASTAIN® ABC kit (Vector labs). Nephrin-positive glomerular area was measured in 15-20 glomeruli per animal using Image J software.

Gene Expression

Cardiac, hepatic, and renal total RNA were extracted using RNA Wiz (Ambion) and RNeasy Midi Kit (Qiagen, Valencia, CA), and aortic RNA was extracted with RNeasy Mini Kit (Qiagen). Reverse transcription was performed using TaqMan Reverse Transcription Kit (Applied Biosystems, Branchburg, NJ). Quantitative real-time polymerase chain reaction was performed in duplicate on the iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA) using iQ SYBR Green Supermix (Bio-Rad) using primers as previously described and 50μL total reaction volume.[39] Template cDNA used in the reaction was 2Jg for heart and kidney and 500ng for aorta. Experimental cycle threshold (C_t) values were normalized to β-actin measured on the same plate, and fold differences in gene expression were determined using the $2^{-\ \ Ct}$ method.[44]

Statistics

Data are presented as mean±SEM. Comparisons among treatment groups were performed using ANOVA, with LSD post-hoc testing for between-group pairs. Because not all pair-wise comparisons are of physiologic relevance and normality could not be assumed, we confirmed significant between group differences using nonparametric tests. Wilcoxon signed- rank test and Wilcoxon rank-sum test were used for paired and unpaired comparisons respectively for any non-normally distributed data. Individual drug effect and drug-genotype interaction for injury measures were assessed by multivariable linear regression with genotype, Ang II treatment, and SPL treatment as exposure variables. All statistical analyses were performed using SPSS for Windows version 17.0 (SPSS, Chicago, IL) or the open source statistical package R, version 2.11.1.[45] A two-tailed *P*-value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

REFERENCES

1. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. The Collaborative Study Group. N Engl J Med. 1993; 329:1456–1462. [PubMed: 8413456]

2. Pfeffer MA, McMurray JJ, Velazquez EJ, et al. Valsartan, captopril, or both in myocardial infarction complicated by heart failure, left ventricular dysfunction, or both. N Engl J Med. 2003; 349:1893–1906. [PubMed: 14610160]

3. Mehdi UF, Adams-Huet B, Raskin P, et al. Addition of angiotensin receptor blockade or mineralocorticoid antagonism to maximal angiotensin-converting enzyme inhibition in diabetic nephropathy. J Am Soc Nephrol. 2009; 20:2641–2650. [PubMed: 19926893]

4. Pitt B, Remme W, Zannad F, et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. N Engl J Med. 2003; 348:1309–1321. [PubMed: 12668699]

5. Pitt B, Zannad F, Remme WJ, et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N Engl J Med. 1999; 341:709–717. [PubMed: 10471456]

6. Nagata K, Obata K, Xu J, et al. Mineralocorticoid receptor antagonism attenuates cardiac hypertrophy and failure in low-aldosterone hypertensive rats. Hypertension. 2006; 47:656–664. [PubMed: 16505208]

7. Nagase M, Shibata S, Yoshida S, et al. Podocyte injury underlies the glomerulopathy of Dahl salt-hypertensive rats and is reversed by aldosterone blocker. Hypertension. 2006; 47:1084–1093. [PubMed: 16636193]

8. Du J, Fan YY, Hitomi H, et al. Mineralocorticoid receptor blockade and calcium channel blockade have different renoprotective effects on glomerular and interstitial injury in rats. Am J Physiol Renal Physiol. 2009; 297:F802–F808. [PubMed: 19535572]

9. Fiebeler A, Nussberger J, Shagdarsuren E, et al. Aldosterone synthase inhibitor ameliorates angiotensin II-induced organ damage. Circulation. 2005; 111:3087–3094. [PubMed: 15939810]

10. Lea WB, Kwak ES, Luther JM, et al. Aldosterone antagonism or synthase inhibition reduces end-organ damage induced by treatment with angiotensin and high salt. Kidney Int. 2009; 75:936–944. [PubMed: 19225557]

11. Jaffe IZ, Mendelsohn ME. Angiotensin II and aldosterone regulate gene transcription via functional mineralocortocoid receptors in human coronary artery smooth muscle cells. Circ Res. 2005; 96:643–650. [PubMed: 15718497]

12. Mazak I, Fiebeler A, Muller DN, et al. Aldosterone potentiates angiotensin II-induced signaling in vascular smooth muscle cells. Circulation. 2004; 109:2792–2800. [PubMed: 15159288]

13. Luther JM, Wang Z, Ma J, et al. Endogenous aldosterone contributes to acute angiotensin II-stimulated plasminogen activator inhibitor-1 and preproendothelin-1 expression in heart but not aorta. Endocrinology. 2009; 150:2229–2236. [PubMed: 19106220]

14. Makhanova N, Lee G, Takahashi N, et al. Kidney function in mice lacking aldosterone. Am J Physiol Renal Physiol. 2006; 290:F61–F69. [PubMed: 16118390]

15. Fiebeler A, Schmidt F, Muller DN, et al. Mineralocorticoid receptor affects AP-1 and nuclear factor-kappab activation in angiotensin II-induced cardiac injury. Hypertension. 2001; 37:787–793. [PubMed: 11230374]

16. Blasi ER, Rocha R, Rudolph AE, et al. Aldosterone/salt induces renal inflammation and fibrosis in hypertensive rats. Kidney Int. 2003; 63:1791–1800. [PubMed: 12675855]

17. Rocha R, Martin-Berger CL, Yang P, et al. Selective aldosterone blockade prevents angiotensin II/salt-induced vascular inflammation in the rat heart. Endocrinology. 2002; 143:4828–4836. [PubMed: 12446610]

18. Brilla CG, Matsubara LS, Weber KT. Anti-aldosterone treatment and the prevention of myocardial fibrosis in primary and secondary hyperaldosteronism. J Mol Cell Cardiol. 1993; 25:563–575. [PubMed: 8377216]

19. Young M, Funder JW. Eplerenone, but not steroid withdrawal, reverses cardiac fibrosis in deoxycorticosterone/salt-treated rats. Endocrinology. 2004; 145:3153–3157. [PubMed: 15059952]

20. Neves MF, Amiri F, Virdis A, et al. Role of aldosterone in angiotensin II-induced cardiac and aortic inflammation, fibrosis, and hypertrophy. Can J Physiol Pharmacol. 2005; 83:999–1006. [PubMed: 16391708]

21. Funder JW. Mineralocorticoid receptors and cardiovascular damage: it's not just aldosterone. Hypertension. 2006; 47:634–635. [PubMed: 16505209]

22. Wang H, Shimosawa T, Matsui H, et al. Paradoxical mineralocorticoid receptor activation and left ventricular diastolic dysfunction under high oxidative stress conditions. J Hypertens. 2008; 26:1453–1462. [PubMed: 18551023]

23. Mulder P, Mellin V, Favre J, et al. Aldosterone synthase inhibition improves cardiovascular function and structure in rats with heart failure: a comparison with spironolactone. Eur Heart J. 2008; 29:2171–2179. [PubMed: 18586661]

24. Matsui Y, Jia N, Okamoto H, et al. Role of osteopontin in cardiac fibrosis and remodeling in angiotensin II-induced cardiac hypertrophy. Hypertension. 2004; 43:1195–1201. [PubMed: 15123578]

25. Rocha R, Stier CT Jr. Kifor I, et al. Aldosterone: a mediator of myocardial necrosis and renal arteriopathy. Endocrinology. 2000; 141:3871–3878. [PubMed: 11014244]

26. Gomez-Sanchez EP, Gomez-Sanchez CM, Plonczynski M, Gomez-Sanchez CE. Aldosterone synthesis in the brain contributes to Dahl salt-sensitive rat hypertension. Exp Physiol. 2010; 95:120–130. [PubMed: 19837774]

27. Shibata S, Nagase M, Yoshida S, et al. Modification of mineralocorticoid receptor function by Rac1 GTPase: implication in proteinuric kidney disease. Nat Med. 2008; 14:1370–1376. [PubMed: 19029984]

28. Fujita T. Mineralocorticoid receptors, salt-sensitive hypertension, and metabolic syndrome. Hypertension. 2010; 55:813–818. [PubMed: 20176994]

29. Grossmann C, Gekle M. New aspects of rapid aldosterone signaling. Mol Cell Endocrinol. 2009; 308:53–62. [PubMed: 19549592]

30. Rafiq K, Nakano D, Ihara G, et al. Effects of mineralocorticoid receptor blockade on glucocorticoid-induced renal injury in adrenalectomized rats. J Hypertens. 2011; 29:290–298. [PubMed: 21243738]

31. Brem AS, Morris DJ, Ge Y, et al. Direct fibrogenic effects of aldosterone on normotensive kidney: an effect modified by 11β-HSD activity. American Journal of Physiology Renal Physiology. 2010; 298:F1178–F1187. [PubMed: 20200098]

32. Lee SH, Yoo TH, Nam BY, et al. Activation of local aldosterone system within podocytes is involved in apoptosis under diabetic conditions. Am J Physiol Renal Physiol. 2009; 297:F1381–F1390. [PubMed: 19710242]

33. Terada Y, Kobayashi T, Kuwana H, et al. Aldosterone stimulates proliferation of mesangial cells by activating mitogen-activated protein kinase 1/2, cyclin D1, and cyclin A. J Am Soc Nephrol. 2005; 16:2296–2305. [PubMed: 15975997]

34. Rautureau Y, Paradis P, Schiffrin EL. Cross-talk between aldosterone and angiotensin signaling in vascular smooth muscle cells. Steroids. 2011

35. Remuzzi G, Perico N, Macia M, Ruggenenti P. The role of renin-angiotensin-aldosterone system in the progression of chronic kidney disease. Kidney Int Suppl. 2005:S57–S65. [PubMed: 16336578]

36. Eddy AA, Fogo AB. Plasminogen activator inhibitor-1 in chronic kidney disease: evidence and mechanisms of action. J Am Soc Nephrol. 2006; 17:2999–3012. [PubMed: 17035608]

37. Hollenberg NK. Implications of species difference for clinical investigation: studies on the renin-angiotensin system. Hypertension. 2000; 35:150–154. [PubMed: 10642291]

38. Lee G, Makhanova N, Caron K, et al. Homeostatic responses in the adrenal cortex to the absence of aldosterone in mice. Endocrinology. 2005; 146:2650–2656. [PubMed: 15731365]

39. Weisberg AD, Albornoz F, Griffin JP, et al. Pharmacological inhibition and genetic deficiency of plasminogen activator inhibitor-1 attenuates angiotensin II/salt-induced aortic remodeling. Arterioscler Thromb Vasc Biol. 2005; 25:365–371. [PubMed: 15576638]

40. Quaschning T, Ruschitzka F, Shaw S, Luscher TF. Aldosterone receptor antagonism normalizes vascular function in liquorice-induced hypertension. Hypertension. 2001; 37:801–805. [PubMed: 11230376]

41. Ma J, Weisberg A, Griffin JP, et al. Plasminogen activator inhibitor-1 deficiency protects against aldosterone-induced glomerular injury. Kidney Int. 2006; 69:1064–1072. [PubMed: 16528256]

42. Dunn SR, Qi Z, Bottinger EP, et al. Utility of endogenous creatinine clearance as a measure of renal function in mice. Kidney Int. 2004; 65:1959–1967. [PubMed: 15086941]

43. Collins TJ. ImageJ for microscopy. Biotechniques. 2007; 43:25–30. [PubMed: 17936939]

44. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods. 2001; 25:402–408. [PubMed: 11846609]

45. R Development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing; Vienna, Austria: 2010.
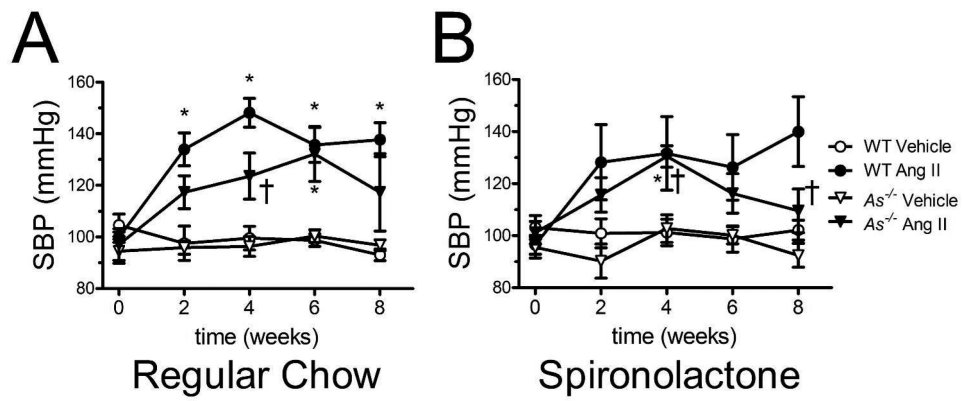
Figure 1.
Systolic blood pressure (SBP) during regular chow (A) and spironolactone chow (B).
Angiotensin (Ang) II increased SBP significantly in wild-type (WT) (○) and aldosterone
synthase-deficient ($As^{-/-}$) (▼) mice, but the blood pressure response was attenuated in $As^{-/-}$.
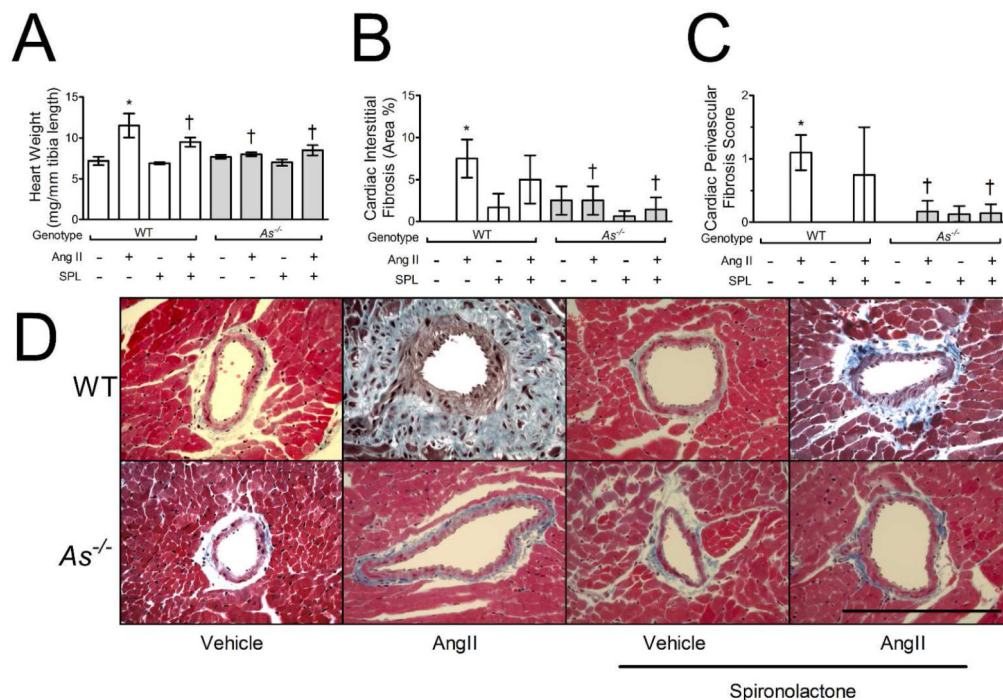*P<0.05 vs. WT-vehicle; †P<0.05 vs. WT-Ang II.

Figure 2.

Angiotensin (Ang) II increased cardiac mass (A) and caused cardiac interstitial fibrosis (B) and perivascular fibrosis (C) in wild-type (WT) mice. Cardiac hypertrophy was similarly abrogated in spironolactone (SPL)-treated and aldosterone synthase-deficient ($As^{-/-}$) mice. Interstitial and perivascular fibrosis were attenuated and not significantly increased in SPL-treated WT mice and were prevented in aldosterone synthase-deficient ($As^{-/-}$) mice. Representative images of cardiac perivascular fibrosis are shown for each treatment group (D); Masson-trichrome (400×), scale bar = 200μm. *P<0.05 vs. WT-vehicle; †P<0.05 vs. WT-Ang II.
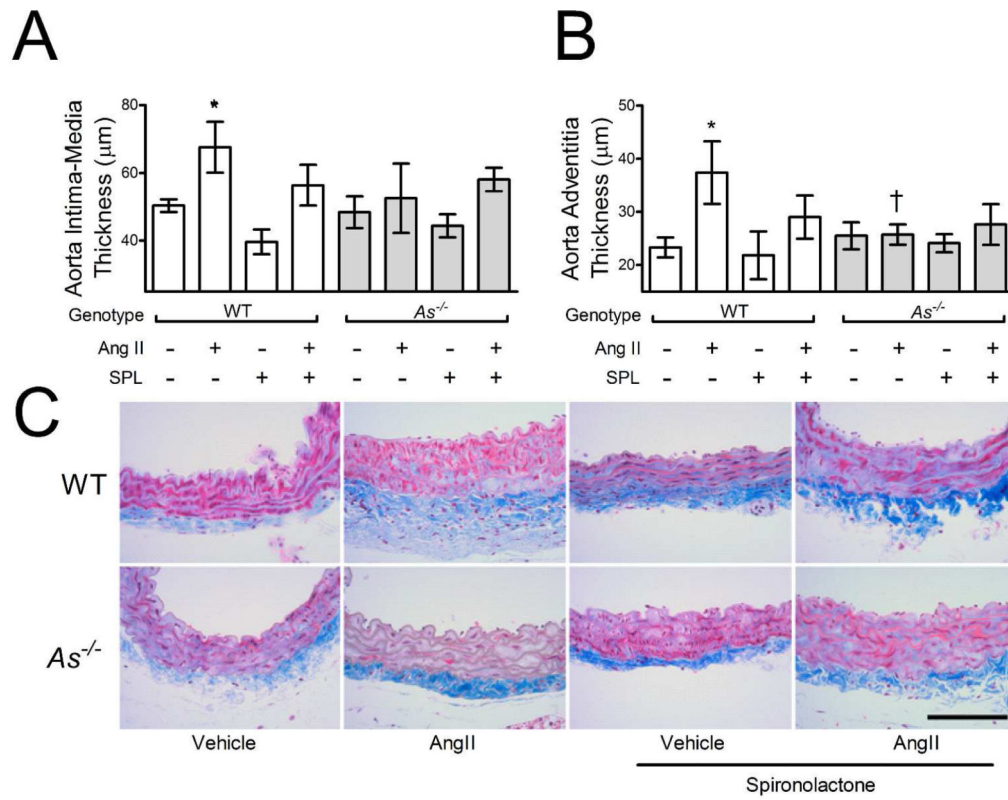
Figure 3.
Angiotensin (Ang) II increased aortic intima-media thickness (A) and adventitial thickness
(B) in vehicle-treated wild-type (WT), but not in spironolactone (SPL)-treated or
aldosterone synthase-deficient ($As^{-/-}$) mice. Representative images are provided (C);
Masson- trichrome stain (400×), scale bar =100μm. *P<0.05 vs. WT-vehicle, †P<0.05 vs.
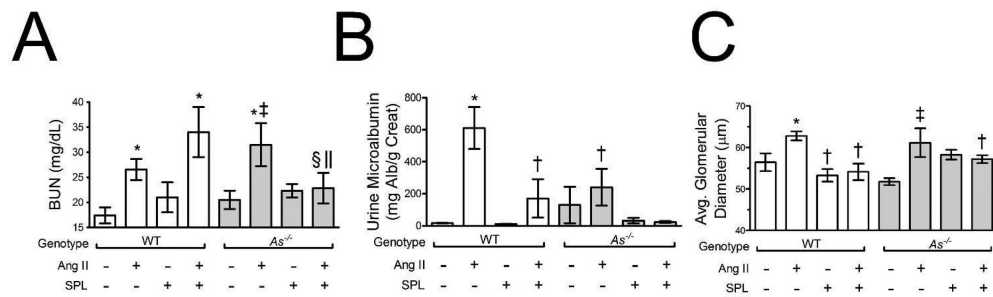WT-Ang II.

Figure 4.
Angiotensin (Ang) II caused an increase in BUN (A), albuminuria (B), and glomerular enlargement (C) in wild-type (WT) mice, and BUN elevation and glomerular enlargement in aldosterone synthase-deficient ($As^{-/-}$) mice. Only combined spironolactone and genetic aldosterone deficiency reduced Ang II-induced BUN elevation. Spironolactone (SPL) prevented albuminuria in Ang II-treated WT mice. $As^{-/-}$ mice were also protected against Ang II-induced albuminuria. SPL prevented glomerular expansion, whereas aldosterone synthase deficiency did not. *$P<0.05$ vs. WT-vehicle; †$P<0.01$ vs. WT-Ang II; ‡$P<0.05$ vs. $As^{-/-}$-Vehicle; □$P<0.05$ vs. WT-Ang II-SPL; §$P<0.05$ vs. $As^{-/-}$-Ang II. BUN, blood urea nitrogen.
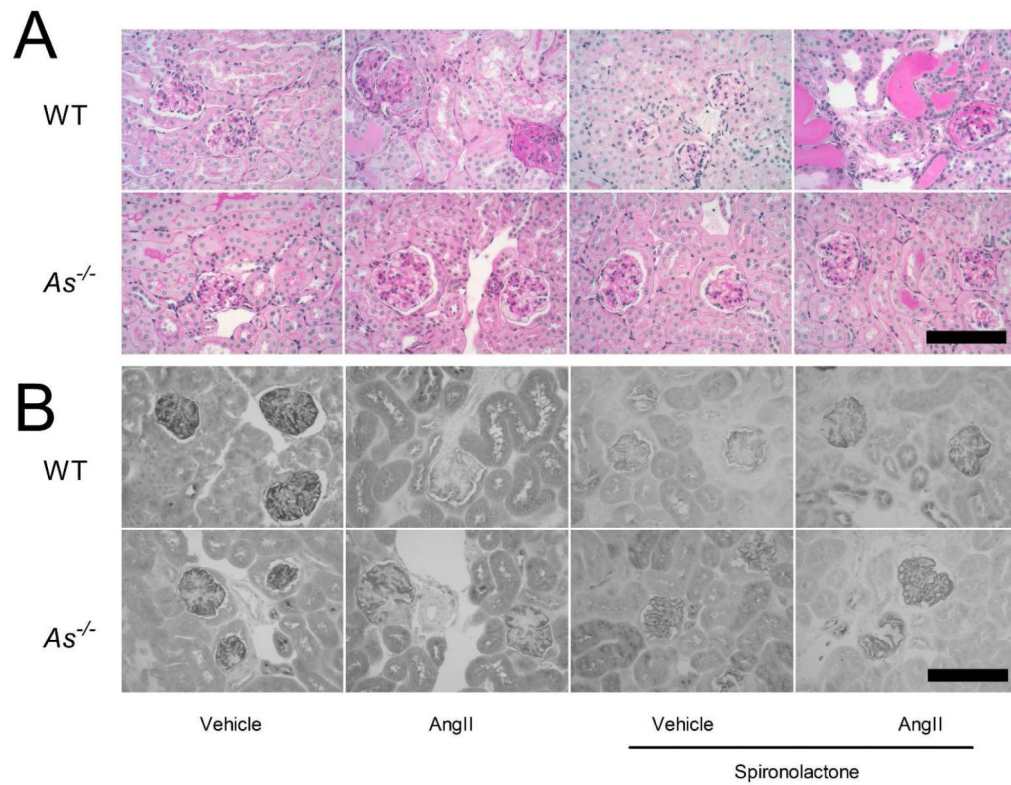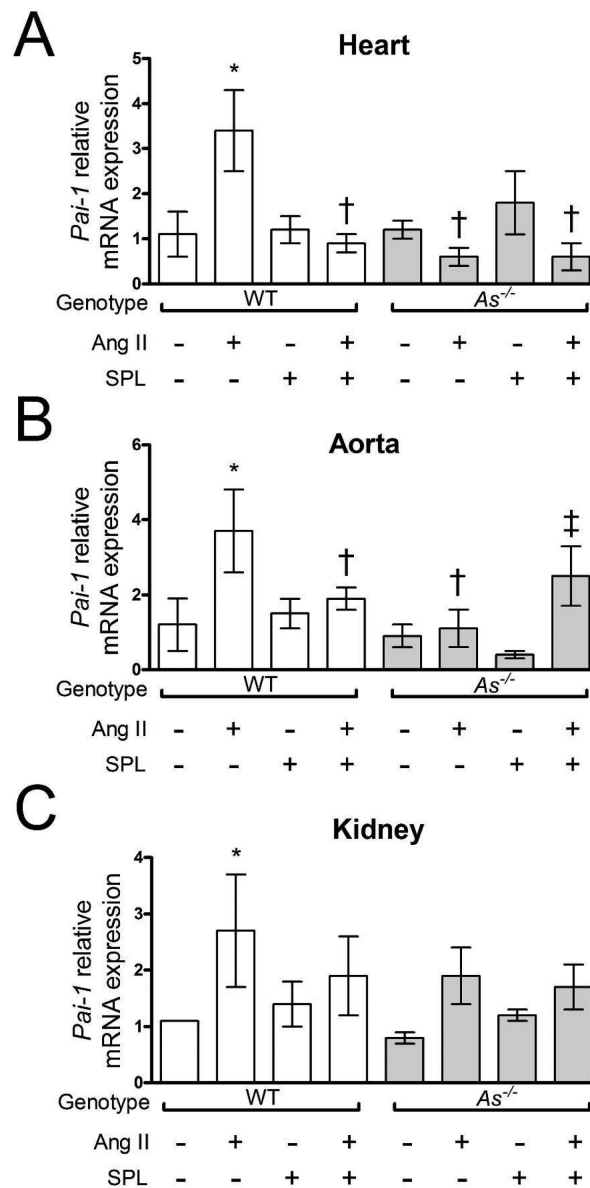
Figure 5.
Representative images of kidney histologic injury are shown for each treatment group (A); Periodic Acid Schiff (PAS) stain. Angiotensin (Ang) II treatment decreased nephrin immunoreactivity (B) in wild-type (WT) and aldosterone synthase deficient (*As*$^{-/-}$) mice. (400×), scale bars = 100μm.

Figure 6.
Angiotensin (Ang) II treatment increased plasminogen activator inhibitor (*Pai*)-*1* mRNA levels in cardiac (A), renal (B), and aortic (C) tissue in wild-type (WT) mice. Spironolactone (SPL) or genetic aldosterone synthase deficiency (*As*^{-/-}) prevented this increase within the heart and aorta, and attenuated the effect within the kidney. *P<0.05 vs. WT-vehicle; †P<0.05 vs. WT-Ang II; ‡P<0.05 vs. *As*^{-/-}-Vehicle-SPL.

Table 1

Physiologic measurements at end of study protocol

	WT-Vehicle	WT Ang II	WT-Vehicle SPL	WT Ang II SPL	As$^{-/-}$-Vehicle	As$^{-/-}$ Ang II	As$^{-/-}$-Vehicle SPL	As$^{-/-}$ Ang II SPL
Body Weight (g)	28.0±1.0	27.8±0.8	29.1±0.3	28.3±1.2	28.2±0.5	26.2±1.3	26.2±0.8	27.8±1.0
Serum Na$^+$ (mEq/L)	147.7±1.1	147.2±0.9	147.5±0.3	147.5±0.5	145.5±0.6	143.0±0.3	145.2±0.7	146.2±1.1
Serum K$^+$ (mEq/L)	5.07±0.25	5.11±0.23	5.48±0.59	5.35±0.05	5.40±0.6	5.73±0.27	5.15±0.10	5.55±0.29
HCO$_3^-$ (mEq/L)	20.1±1.4	24.5±1.7*	21.5±0.68	26.1±5.2*	21.5±1.5	24.0±2.4	21.0±1.4	21.3±1.33
Aldosterone (pg/mL)	373.0±64	484.0±165	233.0±39	883.9±458*	ND#	ND#	ND#	ND#
Corticosterone (ng/mL)	303.7±64	318.8±65	266.5±28.3	314.6±111.8	149.8±23.9	281.2±34.2	247.1±44.8	211.2±62.7
Urine Na*V (μmol/d)	1043±167	4506±1534*	728±235	3776±1607*	777±126	1500±428†	1286±258	2864±1048¶
Urine K*V (μmol/d)	649±100	917±56	633±186	936±130	710±63	776±110	781±92	1494±426*§
Urine Na/K ratio	1.6±0.1	4.7±1.4*	1.1±0.04	4.7±2.5	1.0±0.1	2.2±0.8	1.6±0.2	1.6±0.2†
Urine Alb/Cr (mg/g)	25.9±8.8	610.9±124.5*	11.7±0.9	171.0±120.1†	130.0±113.6	240.7±114.7†	32.5±17.2	23.9±6.0†

Results are mean±SEM

‡P<0.05 vs. WT-vehicle SPL

□P<0.05 vs. As$^{-/-}$-Vehicle SPL

ND, below detectable limit

*
P<0.05 vs. WT-vehicle

†
P<0.05 vs. WT Ang II

§
P<0.05 vs. As$^{-/-}$-Vehicle

¶
P<0.05 vs. WT Ang II SPL

#
P<0.05 vs. all WT treatment groups.