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Hypertension-induced renal fibrosis and spironolactone response vary by rat strain and mineralocorticoid receptor gene expression

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Abstract

Introduction. Aldosterone promotes renal fibrosis via the mineralocorticoid receptor (MR), thus contributing to hypertension-induced nephropathy. We investigated whether MR gene expression influences renal fibrosis and MR antagonist response in a two-kidney, one-clip hypertensive rat model.

Materials and methods. Brown Norway (BN), Lewis, and ACI rats were randomised to spironolactone 20 mg/kg/day or water by gavage, starting four weeks after left renal artery clipping. Blood pressure was measured bi-weekly by tail cuff. After eight weeks of treatment, right kidneys were removed and examined for fibrosis and gene expression. Rats of each strain undergoing no intervention served as controls.

Results. Blood pressure increased similarly among strains after clipping and was unaffected by spironolactone. Hypertension caused the greatest renal fibrosis in BN rats ($p < 0.001$ by ANOVA compared to other strains). Real-time PCR analysis showed greater renal collagen type I and MR gene expression in untreated, hypertensive BN rats (both $p < 0.05$ compared to other strains). Spironolactone attenuated fibrosis, with similar fibrosis among strains of spironolactone-treated rats.

Conclusion. Hypertension-induced renal fibrosis was greatest in rats with the highest MR gene expression. Spironolactone abolished inter-strain differences in fibrosis. Our data suggest that MR genotype may influence aldosterone-induced renal damage, and consequently, renal response to aldosterone antagonism.

Introduction

Aldosterone contributes to hypertension-induced renal damage by promoting renal inflammation, glomerular injury, and abnormal accumulation of fibrillar collagens.¹⁻³ The renal effects of aldosterone are believed to be mediated via the

mineralocorticoid receptor (MR; NR3C2), a member of the nuclear receptor superfamily.⁴ MR antagonism with spironolactone has been shown to provide renal protective effects through mechanisms independent of blood pressure reduction.⁵⁻⁷

There is evidence of inter-patient variability in susceptibility to hypertension-induced renal damage.^{8,9} In addition, we have previously described inter-patient differences in response to MR antagonists.¹⁰ Polymorphisms in genes encoding for proteins involved in the pathogenesis of hypertension-induced organ damage are believed to contribute to the inter-patient variability in target organ complications.¹¹ Similarly, inter-patient differences in drug response may be largely attributed to genetic polymorphisms for proteins involved in drug disposition or pharmacodynamics.¹² Given that the MR is expressed in renal tissue and that MR antagonists attenuate aldosterone-mediated injury, the MR gene is a potential candidate for influencing response to aldosterone and MR antagonists.^{2,4} We hypothesised that aldosterone-mediated effects on the kidney and spironolactone response vary by MR genotype. We exposed three strains of normotensive inbred rats to renovascular hypertension using a two-kidney, one-clip model (Goldblatt model). Using this model, we sought to determine whether MR gene expression is associated with renal fibrosis and attenuation of fibrosis with spironolactone.

Materials and methods

Animals

Experiments were conducted in male Brown Norway (BN/RijHsd), Lewis (Lew/SsNHsd), and ACI (ACI/SegHsd) rats. Brown Norway (BN) and Lewis rats were previously shown to have different MR genotypes, while MR genotype is similar in Lewis and ACI strains.¹³ Differential findings between the BN and Lewis strains would implicate the MR gene as a potential contributing

factor, while differences between the Lewis and ACI strains would implicate the involvement of other genes.

Thirty-two rats of each strain, 10 weeks of age, were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN, USA) and randomised into three groups: (1) clip-untreated (14 rats of each strain); (2) clip-spiro-lactone (14 rats of each strain); and (3) control (four rats of each strain). Control animals were primarily included for blood pressure comparisons to ascertain the effectiveness of renal artery clipping. Animals were housed two per cage in a room with a 12-hour light/dark cycle and free access to standard rat chow (0.3% sodium) and allowed two weeks to adjust after arrival before undergoing surgery. All animal protocols were approved by the Institutional Animal Care Committee at the University of Illinois at Chicago and conducted according to the *Guiding Principles in the Care and Use of Animals* of the American Physiological Society.

Two-kidney, one-clip hypertension

At 12 weeks of age, clip-untreated and clip-spiro-lactone rats were weighed and anaesthetised with ketamine 90 mg/kg plus xylazine 3 mg/kg by intraperitoneal injection. Under aseptic conditions, the abdomen was shaved, and a left lateral abdominal incision exposed the kidney. The left renal artery was freed from the retroperitoneal tissue and dissected from the renal vein. A clip (0.203, 0.214, or 0.229 mm in diameter) constructed from silver wire (MWS Wire Industries, Westlake Village, CA, USA) was placed around the left renal artery. The clip size that constricted renal blood flow without completely occluding the renal artery, as visualised by microscopy, was used. The kidney and artery were placed back *in situ*, and the incision was closed with nylon sutures. Post-operatively, rats were placed on a heating blanket in a clean cage with standard chow and tap water *ad libitum* and treated with buprenorphine 0.1 mg/kg by subcutaneous injection every six hours for two doses. Once sutures were removed, animals were re-housed with their original cage mates.

Blood pressure analysis

Systolic blood pressure was measured before and bi-weekly after clipping by a non-invasive tail cuff system (Model 29-SSP, IITC Inc., Life Science, Woodland Hills, CA, USA). Control rats also underwent bi-weekly blood pressure measurement, beginning at 12 weeks of age. Animals were warmed to 30°C, and the cuff was placed at the base of the tail prior to measurement. Blood pressure measurements were repeated approximately

every two minutes until two readings within 5 mmHg were obtained. All blood pressure measurements were done between 1:00 p.m. and 4:00 p.m. Renal artery clipping was deemed successful if average systolic blood pressure from weeks two through 12 after clipping was ≥ 160 mmHg.

Spiro-lactone treatment

Treatment with 20 mg/kg/day of spiro-lactone (Sigma-Aldrich Corp., St. Louis, MO, USA) by gavage was started four weeks after surgery and continued for eight weeks. Clip-untreated rats were gavaged daily with water, and control animals received no treatment. A spiro-lactone 5 mg/ml suspension, prepared by combining spiro-lactone, 1% methylcellulose, 0.2% Tween-80, and filtered water, was stored at 4°C and shaken well before each use. Rats were weighed weekly, and the spiro-lactone dose was adjusted accordingly.

Urine and tissue collection

At 24 weeks of age, rats were housed individually in metabolic cages with tap water *ad libitum* and without rat chow for 24-hour urine collection. Then, rats were anaesthetised with intraperitoneal ketamine 90 mg/kg plus xylazine 3 mg/kg and weighed. The right (unclipped) kidney was rapidly excised, rinsed, weighed, and frozen in liquid nitrogen. Tissue was stored at -70°C until collagen and gene expression studies.

Hydroxyproline assay

Collagen content of the right kidney was determined by hydroxyproline assay in control rats and five random clip-untreated and clip-spiro-lactone rats of each strain, as previously described.³ Briefly, approximately 40 mg of tissue was excised from the mid region of the right kidney and freeze dried, weighed, cut into small pieces, and washed five times in a solution of 0.1 mol/L NaCl and 5 mmol/L NaHCO₃. Each sample was then hydrolysed in 700 μl of 6N HCl for 16 hours at 110°C, filtered, vacuum dried overnight, and dissolved in deionised water. Hydroxyproline content was determined by the method of Stegemann and Stalder¹⁴ using a colourimetric assay and standard curve of 0 μg to 5 μg hydroxyproline. The data were expressed as μg collagen/mg dry weight, assuming that collagen contains an average of 13.5% hydroxyproline.¹⁵

Aldosterone assay

The total amount of urine produced was recorded, and a well mixed aliquot was preserved with boric acid 10 mg/ml and stored at 4°C for up to one month until analysis. Urinary aldosterone concentrations were determined in duplicate by radio-immunoassay with iodine-125-labelled aldosterone

Table 1

Real-time PCR primer sequences.

Gene	Primer sequence	Product size (bp)	Accession no.
MR	5'-GCCCGGCAAATCTCAACAACACTCAA-3' 5'-TTAGGGAAAGGAACGTCGTGAGCA-3'	235	M36074
Collagen type I	5'-AGCAAAGGCAATGCTGAATCGTCC-3' 5'-TGCCAGATGGTTAGGCTCCTTCA-3'	125	Z78279
GAPDH	5'-TGACTTACCCACGGCAAGTTCAA-3' 5'-ACGACATACTCAGCACCAGCATCA-3'	141	X02231

Key: MR=mineralocorticoid receptor; GAPDH=glyceraldehyde-3-phosphate dehydrogenase.

(Diagnostic Systems Laboratories, Webster, TX, USA) according to the manufacturer's instructions.

RNA isolation and cDNA synthesis

RNA was isolated from tissue taken from the mid section of the right kidney of all control, clip-untreated, and clip-spironolactone rats of each strain using TRIzol[®] Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA (1 µg) was DNase-treated using proprietary reaction buffer and enzyme (Fermentas, Hanover, MD, USA). RNA, DNase, and buffer were incubated at 37°C for 30 minutes. DNase was then heat inactivated at 75°C for 5 minutes. This reaction product was reverse transcribed into cDNA using Oligo(dT) Primer and M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol.

Real-time PCR

cDNA generated by the reverse transcriptase reaction was used as a template in the quantitative real-time PCR assay (qPCR). qPCR primers (table 1) for MR, collagen $\alpha 1$ type I, and the house-keeper gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Quest software (Integrated DNA Technologies, Correlville, IA, USA). The qPCR assay was carried out in triplicate using an ABI 7900HT detection system and SYBR[®] Green PCR Mix for ABI PRISM (Applied Biosystems, Foster City, CA, USA). In short, 12.5 µl master mix containing Taq DNA polymerase, reaction buffer, deoxynucleoside triphosphate mix, and SYBR Green I dye was combined with 2.5 µM concentration of appropriate primers and 2 µl of cDNA. The thermocycling protocol was: 50°C for two minutes, denaturation at 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The temperature transition rate was 20°C/second. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by heating the product at 20°C/second to 95°C, cooling it at 20°C/second to 60°C, keeping it at 60°C for 20 seconds, and then slowly heating it at 0.1°C/second to 95°C. Fluorescence was measured through the slow heating phase. The

efficiency of each primer set was calculated with serial dilutions of cDNA in a qPCR assay, and then the primer efficiency was used to calculate the expression ratio of MR and collagen type I relative to GAPDH, as previously described by Pfaffl.¹⁶ The mRNA quantity of 1 µg was normalised to the mRNA quantity of the endogenous control gene GAPDH from the same sample. Specificity of qPCR primers was confirmed using melting curves and gel electrophoresis. As a negative control, total RNA samples were DNase-treated and then put through reverse transcription in which all reagents except for reverse transcriptase were present. The resulting samples did not PCR, confirming that there was no genomic DNA contamination in the samples tested.

Data analysis

Data were compared between groups within each strain (control versus clip-untreated or clip-untreated versus clip-spironolactone) by the Student's unpaired *t*-test. Data were compared among the three strains by one-way analysis of variance (ANOVA), with subsequent pairwise comparisons by the Student's unpaired *t*-test. Power calculations were based on determining a significant difference between strains in attenuation of renal collagen content (collagen content in untreated rats minus collagen content in treated rats) with spironolactone. Including at least five untreated and five treated rats of each strain was estimated to provide 80% power to detect a 30 mcg/mg difference in renal collagen regression among strains with an α of 0.05 (two-tailed test), assuming a pooled standard deviation of 10 mcg/mg.

Results

Systolic blood pressure

Renal artery clipping successfully increased systolic blood pressure without causing renal infarction in 20 BN rats (nine clip-untreated and 11 clip-spironolactone rats), 21 Lewis rats (10 clip-untreated and 11 clip-spironolactone rats), and 21 ACI rats (nine clip-untreated and 12 clip-spironolactone rats). Blood pressure increased similarly

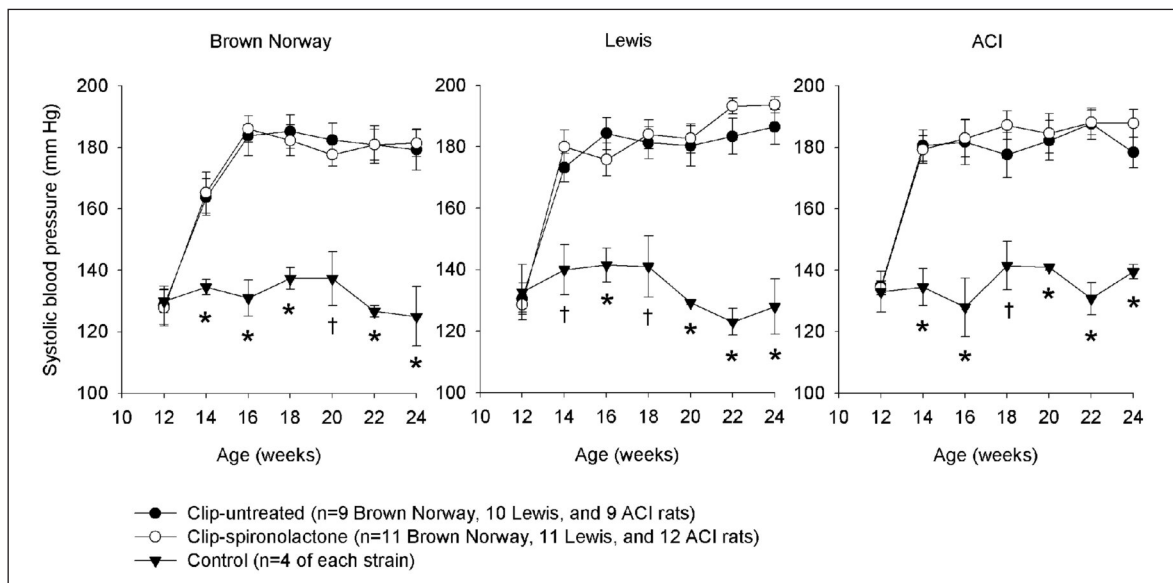


Figure 1
Systolic blood pressures measured bi-weekly using a non-invasive tail cuff system in control, clip-untreated, and clip-spiro-nolactone rats of the Brown Norway, Lewis, and ACI strains. Clip rats underwent a two-kidney, one-clip procedure at 12 weeks of age. Clip-spiro-nolactone rats received spiro-nolactone 20 mg/kg/day by gavage from weeks 16 to 24. Values represent mean±SEM. *p < 0.01, †p < 0.05 for clip-untreated and clip-spiro-nolactone groups compared to control.

Strain	Aldosterone (ng/day)	Body weight (gm)	Right kidney/left kidney weight
Brown Norway			
Control (n=4)	7.9±3.5	281±11	1.05±0.03
Clip-untreated (n=9)	20.5±9.3*	298±27	1.20±0.13*
Clip-spiro-nolactone (n=11)	17.0±7.3*	304±22*	1.37±0.35*
Lewis			
Control (n=4)	8.9±2.4	351±29	1.02±0.02
Clip-untreated (n=10)	11.0±8.3	355±42	1.48±0.56*
Clip-spiro-nolactone (n=11)	10.1±8.7	313±40	1.43±0.31*
ACI			
Control (n=4)	4.7±0.9	249±6	1.06±0.06
Clip-untreated (n=9)	24.2±15.9*	232±14*	1.25±0.18*
Clip-spiro-nolactone (n=12)	22.1±9.8*	218±20*	1.23±0.17*

Key: Mean±SD; *p < 0.05 versus control rats of the same strain.

among strains, as shown in figure 1. Among clip-untreated rats, the mean±SD systolic blood pressure over the 12-week period following surgery was 182±10 mmHg in BN rats and 182±12 mmHg in both Lewis and ACI rats. Spiro-nolactone had no effect on blood pressure in any of the three strains.

Aldosterone excretion

Aldosterone excretion was greater in clip-untreated BN and ACI, but not Lewis rats, compared to respective controls, as shown in table 2. Aldosterone excretion was greater in ACI (p < 0.05) and tended to be greater in BN (p=0.05) rats compared to Lewis rats

after clipping. Spiro-nolactone had no effect on aldosterone excretion in any of the three strains.

Renal measurements

The weight ratio of the right to left kidney was significantly higher in clip-untreated animals compared to controls of each strain and was not affected by spiro-nolactone (table 2). The weight ratio did not differ among the three strains. However, renal collagen content differed significantly among strains of clip-untreated rats, as shown in figure 2 (p < 0.01 by ANOVA). With clipping, renal collagen deposition significantly increased in BN and ACI, but not Lewis rats,

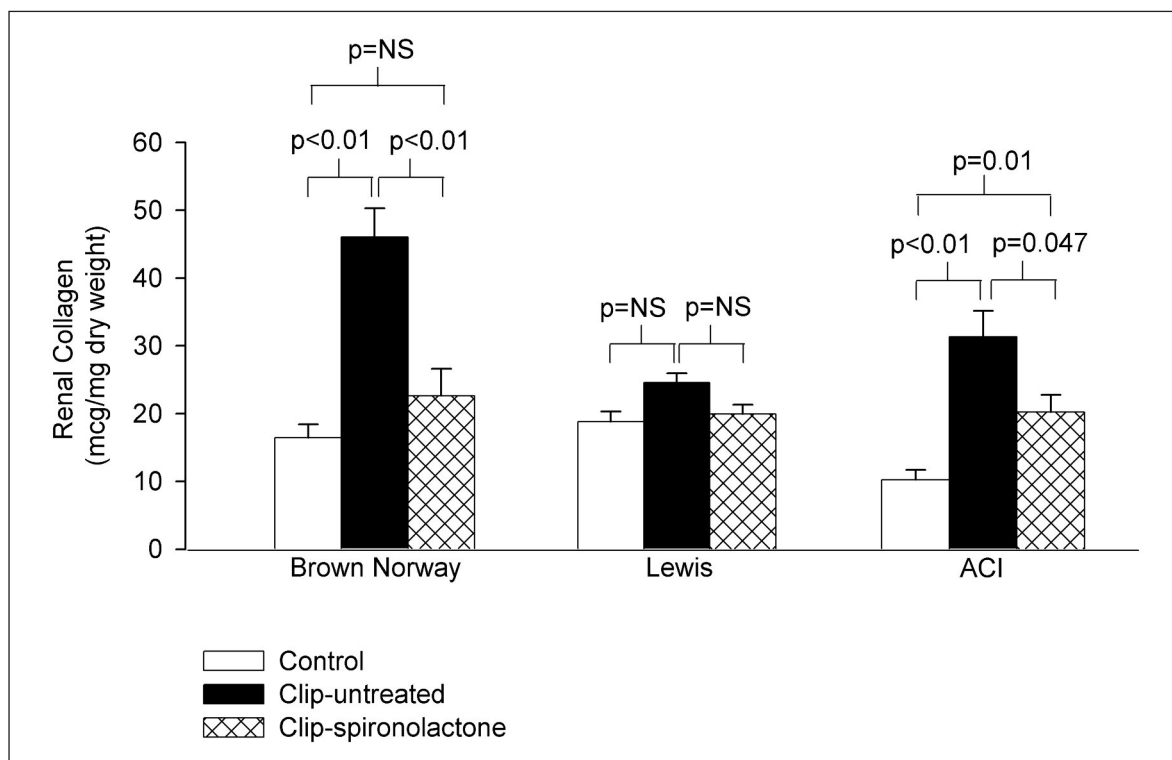


Figure 2

Renal collagen content as measured by hydroxyproline assay in four control, five clip-untreated, and five clip-spiro-lactone rats of the ACI, Brown Norway, and Lewis strains at 24 weeks. Clip-spiro-lactone rats received spiro-lactone 20 mg/kg/day by gavage from weeks 16 to 24. Values represent mean±SEM.

compared to controls. Collagen was greater in clip-untreated BN rats compared to clip-untreated ACI rats ($p < 0.05$) and nearly two-fold greater than in clip-untreated Lewis rats ($p < 0.01$). Spiro-lactone significantly reduced renal fibrosis in BN and ACI strains, although the reduction in ACI rats was of borderline significance. In contrast, spiro-lactone had no effect in Lewis rats. In the BN strain, renal collagen was similar in clip-spiro-lactone and control rats. However, in the ACI strain, renal collagen remained two-fold higher in clip-spiro-lactone rats compared to controls.

Gene expression

Real-time PCR efficiencies were as follows: 0.59 for GAPDH, 0.98 for collagen type I, and 0.70 for MR and in the investigative range from 0.20 to 50 ng of cDNA. Collagen type I gene expression increased significantly in the right renal tissue after clipping in BN and ACI, but not Lewis rats (figure 3). Clip-untreated BN rats had the greatest collagen gene expression ($p < 0.01$ compared to the other two strains). Spiro-lactone treatment tended to attenuate the increase in collagen type I gene expression in BN and ACI rats, although

the difference between clip-untreated versus treated rats did not reach statistical significance.

Renal MR gene expression differed among strains of control rats ($p < 0.01$), as shown in figure 4, with the greatest MR gene expression in BN rats. MR gene expression increased significantly with clipping in BN rats, with greater MR gene expression in this strain compared to both Lewis ($p < 0.01$) and ACI ($p < 0.01$) rats. Spiro-lactone-treated BN rats had lower MR gene expression relative to GAPDH compared to clip-untreated rats (0.026 ± 0.002 versus 0.056 ± 0.001 , $p = 0.04$). MR gene expression did not differ between treated and untreated rats of the other two strains.

Discussion

The two-kidney, one-clip model is a renovascular hypertension model in which reduced renal perfusion leads to activation of the renin-angiotensin system, increased aldosterone secretion from the adrenal gland, and sustained elevations in blood pressure. Using this model, we found significant hypertension-induced increases in renal collagen deposition and gene expression in BN and ACI,

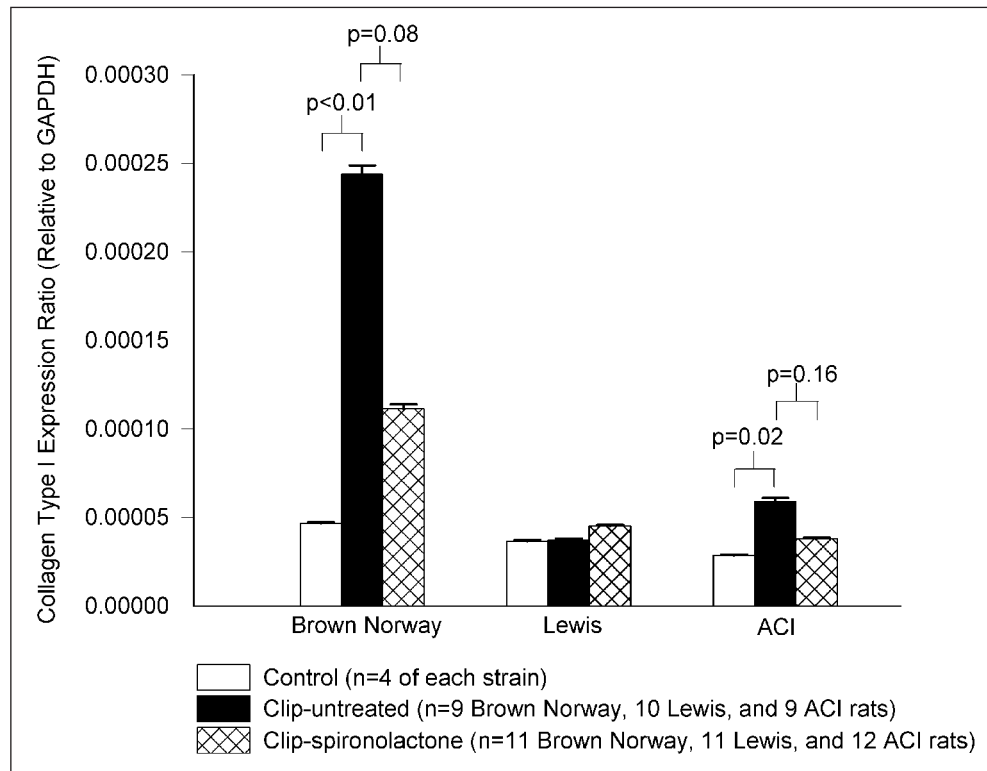


Figure 3 Renal collagen type I gene expression relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in control, clip-untreated, and clip-spirolactone rats of each strain. Values represent mean±SEM.

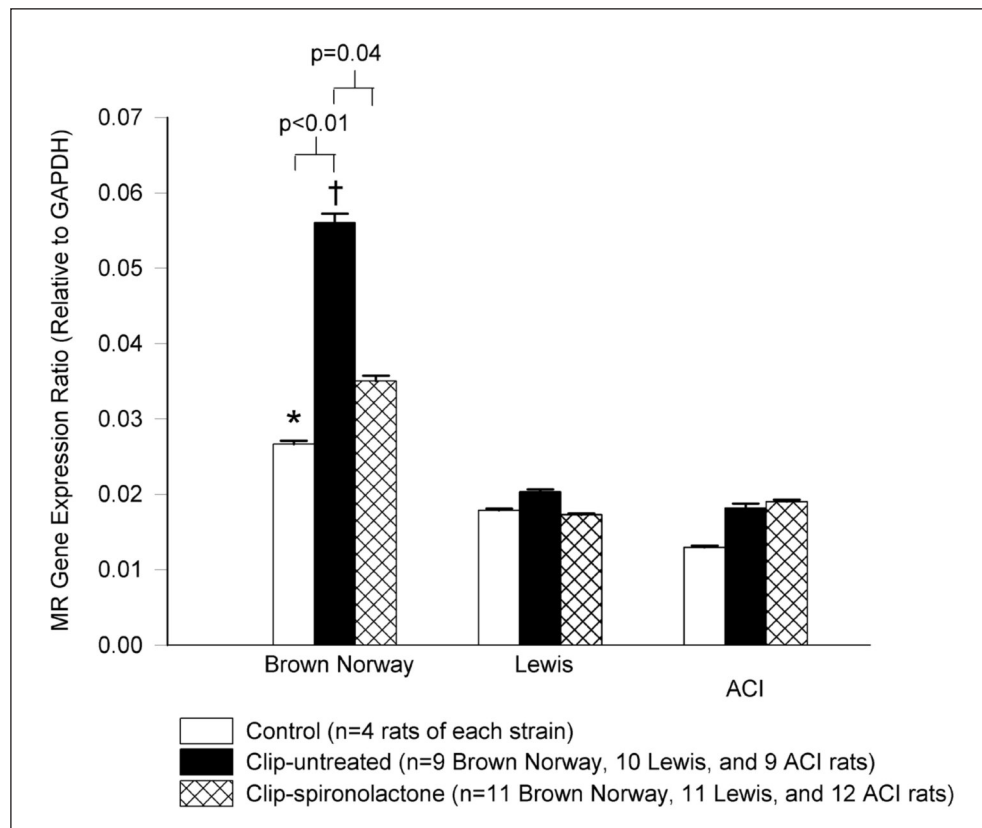


Figure 4 Renal mineralocorticoid receptor gene expression relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in control, clip-untreated, and clip-spirolactone rats of each strain. Values represent mean±SEM. *p < 0.01 compared to control rats of the Lewis and ACI strains; †p < 0.01 compared to clip-untreated rats of the Lewis and ACI strains.

but not Lewis rats. BN rats had the greatest fibrosis and collagen gene expression. Aldosterone antagonism attenuated renal fibrosis in the BN strain, and to a lesser extent in the ACI strain, without affecting blood pressure. Overall, our data suggest that BN rats are particularly prone to hypertension-induced renal damage. This is consistent with findings from other investigators.^{17,18} Our data also suggest that BN rats may derive particular benefit, in terms of renal protection, from aldosterone antagonism. To our knowledge, this has not been previously reported.

Our findings of differential hypertension-induced renal damage and spironolactone response among inbred rat strains with similar blood pressures and maintained under similar conditions suggest that genetic factors contributed to observed inter-strain differences. There are a number of potential candidate genes underlying the differential renal effects. We chose to focus on the MR gene, given that it is the target site for aldosterone and aldosterone antagonists. In addition, other investigators have described differing MR genotypes between BN and Lewis rats.¹³ Greater MR gene expression in renal tissue of BN rats compared to the other strains in our study implicates the MR gene as a contributor to the inter-strain differences in renal fibrosis and spironolactone response.

Increased MR gene expression after renal artery clipping in the BN strain in our study is consistent with previous observations. Specifically, aldosterone has been shown to significantly increase MR mRNA concentration *in vitro*.¹⁹ Similarly, increased MR gene expression has been observed in both post-myocardial infarction and heart failure rat models.^{20,21} These findings are consistent with the MR functioning as a ligand-inducible transcription factor, a characteristic of nuclear receptors.²² In light of these data, perhaps what is most striking about our findings is the absence of increased MR gene expression in the presence of elevated aldosterone in the ACI strain, thereby suggesting that there may be inter-strain variability in the degree of aldosterone-mediated upregulation of MR gene expression. In particular, our data suggest that there may be inter-strain differences in the ligand-binding domain of the MR.

Spironolactone suppressed the rise in MR gene expression in BN rats. This finding is also in line with previous reports, in that attenuation of MR gene expression levels with MR antagonist therapy has been demonstrated in both post-infarct and heart failure rat models.^{20,21} While the exact

mechanism by which spironolactone exerts its effects is unclear, data from our study and others suggest that spironolactone may inhibit hypertension-induced renal fibrosis by modulating MR gene expression levels.²⁰

We observed some attenuation of renal fibrosis among spironolactone-treated hypertensive ACI rats, even though MR gene expression was not increased in this strain. This is possibly due to spironolactone's suppression of aldosterone-induced fibrosis mediated through non-genomic pathways. In support of this possibility, aldosterone has been shown to induce rapid (non-genomic) cellular responses resulting in collagen deposition.²³ These rapid effects were inhibited with MR blockade.²³ Another possibility is that spironolactone, which lacks specificity for the MR, inhibits renal injury mediated through other nuclear hormone receptors. In particular, spironolactone has potent anti-androgen effects.²⁴ There is evidence that testosterone stimulates fibrosis in the kidney.^{25,26} The pro-fibrotic effects of aldosterone could potentially be attenuated by androgen receptor blockade with spironolactone.

Of note, neither aldosterone concentration nor renal fibrosis increased significantly in Lewis rats with renal artery clipping. In contrast, hypertension resulted in increases in both aldosterone excretion and renal fibrosis in the BN and ACI strains. One potential explanation for the lack of hypertension-induced renal fibrosis in the Lewis strain is that this strain had a diminished aldosterone response to renin-angiotensin system stimulation. However, this explanation is speculative since renin concentrations were not measured in the current study.

There are some additional limitations to our study that should be addressed. First, untreated rats were gavaged with water, rather than the vehicle used in the spironolactone preparation. As such, we are not able to account for any potential pharmacologic effects due to the vehicle versus spironolactone. In addition, we did not measure serum or urinary electrolytes in the current study. Thus, whether the effects of aldosterone or MR blockade on electrolyte homeostasis varied by rat strain or gene expression is unknown.

Our findings of greater MR gene expression and renal fibrosis in BN versus ACI rats, despite similar aldosterone concentrations between strains, suggest that the MR gene is involved in determining susceptibility to renal injury from hypertension. If associations between the MR gene and

predisposition to renal disease are identified in humans, then knowledge of MR genotype could have important clinical implications. Specifically, information about MR genotype may be useful in identifying patients at high risk for hypertension-induced renal damage, in whom, more aggressive blood pressure lowering, and potentially therapy with an MR antagonist, may be instituted.

Conclusion

In summary, renovascular hypertension produced differential renal fibrosis in three strains of inbred rats. The strain with the greatest MR gene expression had the greatest renal collagen content and collagen gene expression. Treatment with spironolactone abolished the excessive fibrosis in the rat strain with elevated MR expression. On the other hand, spironolactone only partially attenuated fibrosis in the rat strain without an aldosterone-mediated increase in MR gene expression. These data suggest that MR genotype may influence the degree of MR gene expression and renal damage in renin-dependent hypertension, and consequently, the renoprotective effect of MR antagonism.

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