Abstract
Transgenic rats with inducible expression of the mouse Ren2 gene were used to elucidate mechanisms leading to the development of hypertension and renal injury. Ren2 transgene activation was induced by administration of a naturally occurring aryl hydrocarbon, indole-3-carbinol (100 mg/kg/day by gastric gavage). Blood pressure and renal parameters were recorded in both conscious and anesthetized (butabarbital sodium; 120 mg/kg IP) rats at selected time-points during the development of hypertension. Hypertension was evident by the second day of treatment, being preceded by reduced renal sodium excretion due to activation of the thiazide-sensitive sodium-chloride co-transporter. Renal injury was evident after the first day of transgene induction, being initially limited to the pre-glomerular vasculature. Microalbuminuria and tubulointerstitial injury developed once hypertension was established. Chronic treatment with either hydrochlorothiazide or an AT1 receptor antagonist normalized sodium reabsorption, significantly blunted hypertension and prevented renal injury. Urinary aldosterone excretion was increased ~20 fold, but chronic mineralocorticoid receptor antagonism with spironolactone neither restored natriuretic capacity nor prevented hypertension. Spironolactone nevertheless ameliorated vascular damage and prevented albuminuria. This study finds activation of sodium-chloride co-transport to be a key mechanism in angiotensin II-dependent hypertension. Furthermore, renal vascular injury in this setting reflects both barotrauma and pressure-independent pathways associated with direct detrimental effects of angiotensin II and aldosterone.
or sodium excretion blunt the hypertensive response to Ren2 activation [15,16].

We find that induction of hypertension in the cyp1a1-Ren2 TGR causes sustained activation of the thiazide-sensitive co-transporter by angiotensin II in the distal tubule. Renal injury is predominantly vascular, precedes the development of hypertension and may involve mineralocorticoid-dependent pathways.

**Results**

Systolic blood pressure and diastolic blood pressure increased significantly during the 24 hours following the second dose of I3C (Figure 1). Over the experimental time-course, systolic increased proportionally more than diastolic blood pressure and pulse pressure was therefore increased. Heart rate fell significantly during the period of transgene induction, consistent with an intact baroreceptor reflex. The day-night cycle of locomotor activity was unaffected by RAAS activation. The statistically significant periodicity of blood pressures, heart rate and activity was approximately circadian during the baseline period. Analysis was performed on de-trended data from the hypertensive period and persistency of circadian rhythmicity was observed.

**Activation of the cyp1a1-Ren2 transgene promotes anti-natriuresis**

Under anaesthesia, we observed a progressive increase in mean arterial blood pressure (Figure 2A, ANOVA P<0.001) with

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**Figure 1. Telemetry data from Cyp1a1-Ren2 transgenic rats.** Recordings were made in rats (n = 5) over a baseline period and following 7 consecutive days of indole-3-carbinol administration (I3C: Shaded area). A) locomotor activity; B) Blood pressure (BP), with systolic as a solid line and diastolic as a dashed line; and C) HR. Data are hourly means, smoothed with a 5-point rolling average and shown without error bars for clarity. The vertical lines represent the midnight time point. I3C was administered at 10am. doi:10.1371/journal.pone.0036311.g001
repeated doses of I3C, with a small but significant rise being observed on day 2 of Ren2 induction. Glomerular filtration rate rose significantly, at least until day 4 (Figure 2B; ANOVA $P<0.01$) but then fell back to control levels. Effective renal plasma flow (Table 1) was stable. In consequence, filtration fraction was elevated only on day 4 and renal vascular resistance only on day 8 (Table 1). Transgene induction reduced natriuretic capacity (Figure 2C; ANOVA $P<0.001$), with sodium excretion falling to $\sim 50\%$ of control values by day 2. There was a significant linear trend toward hypokalaemia ($P<0.05$), despite which fractional potassium excretion remained robust (Table 1).

In conscious rats, sodium intake (initially $2.38 \pm 0.11$ mmol/24 h), declined over the induction period but this was not statistically significant until the final day ($1.38 \pm 0.17$ mmol/24 h; $P<0.01$). Body weight was found not to change significantly, with end-weight being $96.5 \pm 1.2\%$ of start weight. 24 h urinary aldosterone excretion was increased $\sim 20$ fold over the induction period (Figure 3 ANOVA $P<0.001$) and we therefore focused our experiments on the aldosterone-sensitive distal nephron (ASDN). Sodium-chloride co-transport (NCC, Slc12a3) and the epithelial sodium channel (ENaC, Scnn1) account for the majority of sodium transport in the ASDN and the contribution of each was assessed pharmacologically. At baseline, thiazide-sensitive transport was responsible for the reabsorption of $\sim 5\%$ of the filtered sodium load. This increased progressively during Ren2-transgene induction (Figure 4A; ANOVA $P<0.01$), as did abundance of total NCC protein relative to GAPDH (Figure 4B; ANOVA $P<0.001$). A positive correlation (Pearson $r=0.60$; $P<0.01$) was observed between NCC protein abundance and thiazide-sensitive sodium reabsorption. An amiloride-sensitive pathway reabsorbed $\sim 2\%$ of the filtered sodium in non-induced rats. There was a slight initial increase in both amiloride-sensitive sodium reabsorption (Figure 4C) and in the abundance of $\alpha$ENaC relative to GAPDH (Figure 4D). Although neither reached statistical significance, both sets of data suggest a similar trend- a transient rise, with both back to baseline by day 8.

The role of NCC-mediated sodium reabsorption was assessed by chronically administering thiazide during transgene induction. The blood pressure increase was significantly attenuated (Figure 4E), but still remained significantly higher than control animals. The partial rescue of the hypertensive phenotype was associated with a large increase in fractional sodium excretion (Figure 4F). An acute bolus of hydrochlorothiazide produced no
Areas of tubulointerstitial injury were also hemorrhage into the necrotic foci (Figure 6C). There was low-confluent medial myocyte death, apoptotic nuclear fragments and vascular damage was both more prevalent and severe, with foci of was also rare focal medial myocyte cell death (Figure 6B). No such medial myocyte vacuolation was evident in arcuate and the larger interlobular arteries, indicative of vasospasm (Figure 6A). There was also rare focal medial myocyte cell death (Figure 6B). No such injury was observed in the control group. In rats studied at day 4, vascular damage was both more prevalent and severe, with foci of confluent medial myocyte death, apoptotic nuclear fragments and hemorrhage into the necrotic foci (Figure 6C). There was low-grade mononuclear cell infiltration into the perivascular adventitia. By day 8, the destructive vascular injury was more extensive still (Figure 6D), and fibroid necrosis was also evident in smaller interlobular arteries and afferent arterioles, consistent with our previous data [4]. Areas of tubulointerstitial injury were also observed, which were small and localized mainly in the cortex. There was no histological evidence of malignant phase hypertension, such as “onion-skinning” of the renal arterioles.

Effect of spironolactone or losartan on blood pressure, renal function an microvascular injury

Modest albuminuria developed over the experimental time-course (Figure 5; ANOVA P<0.001) and kidneys were therefore examined for microvascular injury. An ordered categorical scoring was performed for each field of the microvasculature, indicating a significant (X² analysis; ***P<0.001, **P<0.01 versus the control day.

doi:10.1371/journal.pone.0036311.g003

Data are mean± SE. Comparisons were made using one-way ANOVA (P value shown in column), with Bonferroni post-hoc test: * = P<0.05, ** = P<0.01. FF is fractional excretion of sodium and potassium; V is urine flow rate; PNa and PK are the plasma concentration of sodium and potassium; Hct is hematocrit; eRPF and RBF are effective renal plasma and blood flow, respectively; FF is filtration fraction and RVR is renal vascular resistance.

doi:10.1371/journal.pone.0036311.t001

Activation of the cyp1a1-Ren2 transgene causes microvascular injury

The synergistic effect of angiotensin II and sodium intake on blood pressure has long been known but underlying mechanisms are not fully understood. Angiotensin II promotes sodium reabsorption and stimulates the sympathetic nervous system [22] and vascular smooth muscle [23] to angiotensin II.

Hypertension, sodium reabsorption and thiazide-sensitive co-transport

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Three of these rats were examined histologically and there was no evidence of hypertensive vascular injury. Spironolactone had no antihypertensive effect (Figure 7A) and nor did it have any effect on thiazide-sensitive sodium reabsorption (Figure 7B). Mineralocorticoid receptor blockade did, however, prevent the development of albuminuria during transgene induction (albumin excretion in mg/24 h) Ren2 induction alone = 1.16±0.12; Ren2 induction with co-administration of spironolactone = 0.18±0.07; P<0.01). Kidneys from five spironolactone treated rats were examined histologically: in two cases there was no evidence of vascular injury; in the other three, variable fibroid necrosis was observed.

Discussion

In this study we investigated the early renal adaptation to RAAS activation in the cyp1a1-Ren2 TGR. We show that the early phase of hypertension, characterized in this model by circulating angiotensin II in the high physiological range [5], is associated with impaired natriuresis due to activation of the thiazide-sensitive sodium-chloride cotransporter, with little contribution from amiloride-sensitive sodium transport.
Figure 4. Sodium transport pathways in the aldosterone-sensitive distal nephron. A) hydrochlorothiazide-sensitive sodium reabsorption (ΔThiazideNa); B) the expression of the thiazide-sensitive co-transporter protein (NCC) in whole kidney extracts, normalized to that of GAPDH; C)
amiloride-sensitive sodium reabsorption ($\Delta$amilorideNa); D) the expression of the $\alpha$ subunit of the epithelial sodium channel ($\alpha$ENaC) normalized to that of GAPDH. Measurements were made in cyp1a1-Ren2 transgenic rats, on either day 2 ($n=9$), 4 ($n=9$) or 8 ($n=9$) in the induction regimen. Non-induced rats ($n=8$) served as control. E) mean arterial blood pressure (MABP) and F) fractional sodium excretion. Measurements were made in cyp1a1-Ren2 transgenic rats receiving either vehicle (grey bar) or hydrochlorothiazide (hatched bar) by minipump. Data are mean ± SE. Statistical comparisons were made using ANOVA with Bonferroni post-test. $P<0.001$, **$P<0.01$, *$P<0.05$ versus the control group.

doi:10.1371/journal.pone.0036311.g004

Nevertheless, body weight was stable and in the cross-sectional studies plasma sodium was not changed. In this setting we observed an impaired capacity to excrete an intravenous saline load. In the proximal tubule, angiotensin II exerts a biphasic effect on sodium transport, becoming inhibitory at high concentrations [24,25]. In our study, the increase in fractional lithium excretion observed at days 2 and 4 indicates a reduction in proximal tubule sodium reabsorption [26] and localizes the amniatriuresis to more distal nephron segments. Since mice infused chronically with angiotensin II display increased sodium reabsorption in the ASDN [27], we probed the function of this region with inhibitors of the major pathways for sodium transport, ENaC and NCC. Both angiotensin II and aldosterone can stimulate ENaC activity [13,28] but we found the amiloride-sensitive pathway small and not greatly influenced by transgene induction. There was perhaps a trend towards an increase in both amiloride-sensitive transport and $\alpha$ENaC protein abundance but this normalized by day 8. Our protocol ultimately causes malignant hypertension after ~14 days [4]. In contrast, when induction is aimed at producing a non-sodium reabsorption through Slc4A8 by NCC, but recently it has been shown that they also inhibit ENaC activity, uncovered when NCC is inhibited.

Thiazides are considered to be selective inhibitors of transport by NCC, but recently it has been shown that they also inhibit sodium reabsorption through Slc4A8 in the collecting duct [29]. However, the strong positive correlation between NCC abundance and thiazide-sensitive sodium reabsorption suggests that transport through Slc4A8 plays only a minor role in our studies.

In order to be physiologically active, NCC must be phosphorylated on threonine and serine residues in a regulatory domain in the N-terminus [30,31], and it must be correctly trafficked to the apical membrane of the DCT. In our study, we did not measure NCC phosphorylation or define localization to a specific subcellular compartment. Measurements of NCC phosphorylation are increasingly used as a surrogates for those of transport, yet evidence suggests that although phosphorylation is necessary, it is not sufficient, to explain transport activation in cation-chloride co-transporters [32]. Thus, we consider that the increase in total NCC expression, which paralleled the blood pressure rise, was more than sufficient to account for the increased thiazide-sensitive sodium reabsorption observed.

Previous studies have shown that both angiotensin II and aldosterone can independently increase NCC-mediated sodium transport in the DCT [12,33,34,35,36]. Our data show that the activation of NCC occurs via AT1R as losartan caused a natriuresis and fully normalized NCC activity. In contrast, spironolactone, at levels sufficient to achieve complete mineralocorticoid receptor blockade [37], had no effect on thiazide-sensitive sodium reabsorption or blood pressure. MR activation does not therefore appear to be major factor in our model, consistent with some previous studies [3,6,27], but contrasting with others [38]. It is also reported that hypertension per se reduces NCC expression in the apical membrane of the DCT [39]. We cannot discount a countervailing influence of hypertension on NCC activity, but our data indicate that the overall outcome was an angiotensin II-mediated increase in thiazide-sensitive sodium transport.

Chronic infusion of either hydrochlorothiazide or losartan was an effective antihypertensive measure. Both treatments also increased fractional sodium excretion, implying that renal sodium retention is a key hypertensive mechanism in this model. In support of this hypothesis, reciprocal transplantation studies in AT1AR null and wild-type mice show that renal AT1R mediate the chronic hypertensive effects of angiotensin II infusion by promoting renal sodium reabsorption [40]. However, in our study there was a trend towards volume contraction as blood pressure rose, suggesting that the effects of angiotensin II and dietary salt on blood pressure do not necessarily reflect volume expansion here [19,20]. Indeed, other studies indicate that the antihypertensive effect of thiazide diuretics in angiotensin II-dependent hypertension is not exclusively related to depletion of plasma volume [19] and thiazides might have beneficial effects on vascular resistance [41].

Renal microvascular injury

The afferent arteriole, glomerulus and post-glomerular structures were relatively protected from injury throughout the experiment; mild albuminuria developed by day 4 and was further increased coincident with raised renal vascular resistance. Our data suggest that protection from barotrauma reflects maintenance of efficient autoregulation [42]. In contrast, injury to the larger pre-glomerular resistance arteries was evident on the

![Figure 5. 24-hour urinary excretion of albumin.](image-url)
second day, before elevation of blood pressure into the hypertensive range. Renal injury was consistent with vasospasm, and might reflect damaging intermittent surges in systolic blood pressure [43]. These were not visualized in our telemetry analysis but data were only acquired hourly for short periods.

Pressure-independent renal injury, involving both angiotensin II and aldosterone [44,45,46] is also possible. The extent of pressure-independent renoprotection following RAAS inhibition is contentious [47] as AT1R blockade has exacerbated organ damage in some clinical trials [48]. Animal models suggest that transmission of high pressure to the kidney drives vascular remodeling and injury in angiotensin II-dependent hypertension [49,50]. Our data show a protective effect of AT1R blockade, perhaps partially due to losartan’s antihypertensive effect. However, chronic thiazide therapy, which caused a similar fall in blood pressure, did not abolish vascular injury, supporting a direct role for angiotensin II in end-organ injury. The beneficial effects of spironolactone were more clearly independent of blood pressure. Experimental and clinical data suggest that aldosterone, some of which may be synthesized in the kidney itself [51], is directly fibrogenic [52] and can damage renal structures [53]. Aldosterone-induced podocyte injury [54], for example, may explain the beneficial effect of spironolactone on the albuminuria seen here.

Concluding Remarks
Renal, vascular and endocrine measurements have established distinctive aetiologies of hypertensive and renovascular disease following the conditional over-expression of renin in cyp1a1-Ren2 TGR. Our data show that angiotensin II, aldosterone and sodium status act in concert to cause hypertension and renal impairment and underscore the key role of thiazide-sensitive sodium transport in the long-term regulation of blood pressure. Approximately 1/3 of the blood pressure rise was resistant to thiazide or losartan treatment. A similar proportion of hypertension persists in AT1R null mice infused with angiotensin II [46], pointing to non-renal, non-AT1R-mediated mechanisms. These may involve direct, detrimental actions of renin on vascular function [55], sensitization of the vasculature to vasoactive agents [23] and activation of the sympathetic nervous system [56].

Materials and Methods
Ethics Approval
Experiments were performed under a UK Home Office Project License and protocols were approved by The University of Edinburgh. All surgery was performed under anaesthesia (details are given under relevant sections, below) and all efforts were made to minimize suffering.

Experiments were performed on male cyp1a1-Ren2 TGR on a Fischer (F344) background, from a colony maintained in the university’s animal house. Rats, aged 12–14 weeks, were given free access to water and commercial rat chow, containing 0.3% sodium by weight (Special Diet Services, UK) and were maintained under controlled conditions of temperature (21°C ± 1°C), humidity (50 ± 10%) and light/dark (light 7am–7pm).

Blood pressure measurement in conscious rats
Radiotelemetry devices (Model TA 11PAC20, Data Sciences, UK) were implanted into the thoracic aorta of Cyp1a1-Ren2 TGR (n = 5) under ketamine/medetomidine anaesthetic, with buprenorphine used as a reversal agent and analgesic. After recovery, one-week equilibration was given during which restoration of the circadian rhythms for blood pressures, activity and heart rate was confirmed. Experimental data were then recorded and decoded using Art 4.0 software (Data Sciences, UK).

Baseline readings were taken over four consecutive days and, on day 5, the cyp1a1-Ren2 transgene was induced by administering the naturally occurring xenobiotic, indole-3-carbinol (13C; 100 mg/kg/day in vegetable oil), by gastric gavage at 10am; this was repeated on the following 6 days. Data were obtained on the hour, processed to means, smoothed using a 3-point rolling average and...
Renal Function in the cyp1a1-Ren2 Transgenic Rat

Figure 7. Contributions to hypertension of AT1 and mineralocorticoid receptors. A) Mean arterial blood pressure (MABP) and B) lithium clearance (ΔthiazideL) in cyp1a1-Ren2 transgenic rats. Measurements were made in cyp1a1-Ren2 transgenic rats, anaesthetized on day 4 of the experimental regimen. The first control group (n = 6; open bars) received vehicle, the second control group (n = 8; grey bars) received I3C to induce the Ren2 transgene. Experimental groups received I3C and either losartan (n = 6; grey hatched bars) or spironolactone (n = 6; open hatched bars). Data are means ± SE and statistical comparisons were made using ANOVA with Bonferroni post-test. **P < 0.01, ***P < 0.001, *P > 0.05 versus the non-induced control group and other comparisons as stated. doi:10.1371/journal.pone.0036311.g007

Renal function in anaesthetized rats

Separate cohorts of cyp1a1-Ren2 TGRs received I3C for either 1, 3 or 7 days; the control group (n = 8) received an equivalent volume of vehicle over a 7-day period. Time-points are designated as control (n = 8), day 2 (n = 9); day 4 (= 9) and day 8 (n = 9). In each group, mean arterial blood pressure and renal function were measured twenty-four hours after the final gavage treatment, as follows. Following thiobutabarbital anaesthesia (Inactin, 120 mg/kg IP; Sigma Aldrich UK), a catheter was inserted into the right jugular vein through which a solution, containing (in mmol/l): 120 NaCl, 10 LiCl, 15 NaHCO3 and 5 KCl, was infused (1 ml/h/100 g IV). The infused also contained 0.5% p-amino hippuric acid (PAH) and 0.5% FITC-inulin. A catheter, containing heparin-saline, was placed in the right carotid artery for collection of blood and measurement of mean arterial blood pressure. The bladder was catheterized for collection of urine and a tracheostomy was performed to maintain a clear airway.

One hour after the completion of surgery, rats underwent the following protocol: three consecutive collections of urine were made, each of 60 minutes. A sample (75 µl) of arterial blood was drawn at the start and then after each urine collection period for the measurement of haematocrit, FITC-inulin and PAH.

After the first urine collection, amiloride was administered (2 mg/kg bolus; 2 mg/kg/h infusion). After the second urine collection, hydrochlorothiazide was administered (2 mg/kg bolus; 2 mg/kg/h infusion) alongside the amiloride. The drugs were delivered in a DMSO vehicle (2% v/v); which, in a separate group of rats (n = 7), had no effect on renal function or blood pressure (data not shown). Finally, a 1 ml sample of arterial blood was taken for measurement of plasma electrolytes and the kidneys frozen at −80°C and used for Western blot. Animals were then killed by an overdose of anaesthetic.

Analysis & calculations

The concentration of Na, K and Li in plasma and urine was measured using ion-selective electrodes (Roche ISE, model 9190). The effect of amiloride on sodium excretion was taken as the difference between excretion rates in the first and second urine collections; that of hydrochlorothiazide was taken as the difference between excretion rates in the second and third urine collections. FITC-inulin and PAH concentrations were measured as described [4]. The clearance of PAH was taken as effective renal plasma flow and used to calculate renal blood flow. Renal vascular resistance was taken as the quotient of arterial blood pressure and renal blood flow. Lithium clearance was used as an index of fluid delivery to the end of the proximal tubule [26].

Urine collection in conscious rats

Cyp1a1-Ren2 transgenic rats (n = 8) were housed individually in metabolism cages with free access to powdered rat chow and water. Rats were acclimatized and after a 4-day control period, I3C was administered on each of 7 consecutive days, as described above. Urine was collected every 24-hours. Albumin was measured using commercial assays (Alpha Laboratories Ltd., UK), aldosterone by ELISA [58].

Interventional studies

Hydrochlorothiazide (4 mg/kg/d; n = 6), spironolactone (20 mg/kg/d; n = 6) or losartan (10 mg/kg/d, n = 6) were administered by osmotic minipump (Alzet, Model 2ML1, Charles River UK), implanted on day 0 under isoflurane anaesthesia. A control group of rats (n = 6) received vehicle (50% DMSO, 50% saline) alone. After implant, the Ren2 transgene was induced over a period of three days before being anaesthetized for renal function studies, as described. A three-day induction was selected as a time-point at which significant changes to tubular function were not associated with a decline in renal haemodynamics or major destructive vascular lesions.

Western blot analysis

Total protein was extracted from one kidney by homogenization and differential centrifugation in ice-cold buffer, containing 250 mM sucrose, 10 mM triethanolamine and 2% protease inhibitor cocktail, pH 7.6 (Pierce Protein Research, Thermo-scientific, UK). Protein solubilized in sample buffer (50 µg of total protein per lane) was separated by SDS-PAGE and blotted to PVDF membrane by semi-dry transfer. Immunoblotting was
Histopathologic analysis

Kidneys were immersion-fixed in 10% neutral buffered formalin for 48 h followed by paraffin embedding. Four-micron sections were examined blind by a Consultant Pathologist (C.O.B.). Kidneys from at least three rats per experimental group were examined. For each, 2 complete hemisections of the left kidney were blocked and all vascular profiles in each hemisection examined at 4 different levels in the block. Two were stained with hematoxylin and eosin and two with Periodic Acid Schiff, giving 8 levels per case. Analysis of microvascular injury was performed using an ordered categorical scale according the presence of destructive microvascular arterial or arteriolar lesions characterized by intramural necrosis and/or fibrinoid change. A score of “one” indicates non-confluent necrosis and isolated myocyte death; “two” indicates a single foci of necrosis per section; “three” indicates 2–3 foci; and “four” indicates >3 foci of destructive vascular lesions. Undamaged sections were scored zero.

Statistics

Data are presented as mean ± SE. Comparisons were made using either one- or two-way analysis of variance, as appropriate: post-hoc comparisons were made using the Holm-Sidak test. For scoring of microvascular injury, contingency tables were generated for $X^2$ analysis.

Acknowledgments

We thank Gillian Brooker for expert technical assistance. Ali Ashiek received a University of Edinburgh PhD Studentship; Robert Menzies holds a 4-year PhD studentship from the British Heart Foundation Centre of Research Excellence (CoRE) Award to The University of Edinburgh.

Author Contributions

Conceived and designed the experiments: AA AJH CJK PWF JMJ MB. Performed the experiments: AA RIM LJM COCB MB. Analyzed the data: AA RIM COCB CJK PWF MB. Wrote the paper: PWF MB.


