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Chronic hyperaldosteronism in Cryptochrome null mice induces high-salt- and blood pressure-independent kidney damage in mice

Key words: Aldosterone, Blood pressure, Kidney, Salt
Chronic hyperaldosteronism in Cryptochrome null mice induces high-salt- and blood pressure-independent kidney damage in mice

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Abstract

Although aldosterone plays an essential role in the control of electrolyte and body fluid homeostasis, it also exerts certain pathological effects on the kidney. Several previous studies have attempted to examine these deleterious effects. However, the majority of those studies were done using various injury models, high salt treatment and/or mineralocorticoid administration by which the kidney change observed not only due to aldosterone but also due to prior injury, salt and hypertension. In the present study, we performed study to investigate aldosterone pathological effect on the kidney using mice model with high level of endogenous aldosterone. We used cryptochrome-null (Cry 1, 2 double knockout (DKO)) mice that are characterized by high aldosterone levels and low plasma renin activity, and indicated that even under conditions of normal salt exposure, these mice showed an increase in albumin excretion and kidney tubular injury, a decrease of nephrin expression, and an increase in the production of reactive oxygen species in the absence of hypertension. Exposure to high levels of salt exacerbated the kidney damage observed in these mice. Moreover, we noted that decreasing the blood pressure without blocking the aldosterone action did not provide beneficial effects to the kidney in high-salt-treated Cry 1, 2 DKO mice. Thus, our findings support the hypothesis that aldosterone causes deleterious effects on the kidney independently of high-salt exposure and high blood pressure.

Keywords: aldosterone, blood pressure, kidney, salt.
Introduction

Aldosterone is a mineralocorticoid hormone that is produced in the cortex of the adrenal glands. In physiological conditions, its binding with specific intracellular mineralocorticoid receptors (MRs) in the distal nephron regulates electrolytes and water homeostasis.\(^1\) Thus, aldosterone regulates blood volume and blood pressure.\(^2\) However, aldosterone also contributes to the development of kidney damage, as reported in several studies.\(^3,4\) Primary aldosteronism, which is characterized by high plasma aldosterone levels and low renin activity, is often correlated with an increased risk of kidney damage.\(^5\)

Remnant kidney model in animal with elevated plasma aldosterone level showed hypertension and proteinuria.\(^6\) In another study with increase of mineralocorticoid levels following deoxycorticosterone acetate (DOCA) administration and high-salt treatment in rats subjected to unilateral nephrectomy has been found to induce hypertension, peritubular capillary loss, and tubular interstitial fibrosis.\(^7\) Moreover in rats treated with salt and aldosterone for 28 days, severe kidney damage and hypertension were observed.\(^8\) By using those models, hypertension role in the development of kidney damage could not be ruled out. Studies on several animal models of kidney injury have indicated that the administration of an MR blocker could ameliorate kidney damage independent of blood pressure.\(^9,10\) To further elucidate the aldosterone effect towards the kidney independent of blood pressure, some studies have been performed by decreasing the blood pressure without interfering with the aldosterone effect by using hydralazine treatment\(^11\), whereas other studies have involved the administration of a non-hypotensive dose of an MR blocker\(^12\) or administration of low-dose aldosterone.\(^13\) Even many aldosterone induced renal pathology studies have been done but most of them are using injury models such as subtotal renal ablation and unilateral nephrectomy, mineralocorticoid administration, high salt treatment or combination of these methods by which the aldosterone pathological effect towards the kidney could not be distinguished clearly with the previously existed injury, salt and hypertension.

To study aldosterone induced kidney damage in the absence of prior kidney manipulation, high salt treatment and/or mineralocorticoid administration, we used Cry 1, 2 DKO mice. Cryptochrome is known
to regulate the circadian rhythm. Cry 1, 2 DKO mice show increased mRNA expression and protein levels of 3β hydroxysteroid dehydrogenase, the enzyme involved in the aldosterone synthesis. The enzyme is expressed particularly in the zona glomerulosa where the production of aldosterone is known to exclusively take place. Thus, Cry 1, 2 DKO mice present with high aldosterone levels but low plasma renin activity.

Methods

Animal experiments

In the present study, we used cryptochrome-null (Cry 1, 2 DKO) mice, which characteristically show high plasma aldosterone levels and suppressed renin activity. The development of Cry 1, 2 DKO mice has been described previously. During the course of the study, all the mice were maintained at a stable environmental temperature with a 12-hour light and dark cycle and had free access to water and chow. All the experiment protocols were performed based on the animal experiment guidelines of Kobe University, Kobe, Japan.

We used Cry 1, 2 DKO mice and wild-type littermates as the controls. We divided 12-week-old male mice into the following 6 groups: wild-type mice treated with normal salt (WTNS; n = 17), wild-type mice receiving high-salt treatment (WTHS; n = 16), Cry 1, 2 DKO mice receiving normal salt treatment (CRYNS; n = 13), Cry 1, 2 DKO mice receiving high-salt treatment (CRYHS; n = 20), Cry 1, 2 DKO mice receiving high-salt and spironolactone treatment (CRYSPI; n = 13), and Cry 1, 2 DKO mice receiving high-salt and hydralazine treatment (CRYHYD; n = 6). All the mice underwent the respective treatments for 32 weeks. Normal salt chow refers to 0.2% NaCl content in the chow, whereas high-salt chow refers to 3.15% NaCl content in the chow and 1% NaCl and 0.2% KCl in the drinking water. Spironolactone or hydralazine was administered in the drinking water. The doses of spironolactone (Sigma-Aldrich, St. Louis, MO) and hydralazine (Sigma-Aldrich) were 6mg/kg body weight/day and
25mg/kg body weight/day, respectively. Spironolactone was dissolved in ethanol and then diluted in drinking water.

Blood pressure measurement

Blood pressure was determined in conscious trained mice using a non-invasive computerized automated tail-cuff system (BP-98A, Softron, Tokyo, Japan). The average value of 10 measurements was used for data analysis.

Blood and urine biochemical analysis

Blood was obtained for biochemical analysis after 32 weeks of treatment by cardiac puncture before the mice were sacrificed. A 24-hour urine collection was performed using metabolic cages 1 day before the mice were sacrificed. Creatinine concentrations in the serum and urine were measured using an enzymatic assay (Nescoat VLII CRE Kit; Alfresa Pharma Corp, Osaka, Japan). A commercially available enzyme-linked immunosorbent assay kit was used to measure urine albumin (Bethyl Laboratories, Inc, Montgomery, TX) according to the manufacturer’s instructions. Serum aldosterone was measured using a commercially available enzyme immunoassay kit (Enzo Life Sciences Inc, Plymouth Meeting, PA) according to the protocol described by the manufacturer. Serum and urine electrolyte was measured using ion selective electrode (ISE) and calculated using the Nernst equation.

Gene expression

The total RNAs from the removed kidney were extracted using RNAisoPlus (TaKaRa, Dalian, China). Reverse transcription was performed using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The quantification of the gene expression levels of renin, the MR, 11BHSD2, nephrin, and G6PD was performed using the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) according to the protocol described by the manufacturer. Primers used in this study were as follows: renin (forward: 5'ATGAAGGGGGTCTGTGCGGGTC3'; reverse:
5′ATGTCGGGGAGGGTGGGCACCTG3′), MR (forward: 5′GGAAACAAAGGCTACCACA3′; reverse: 5′AGTGTGGAGGACCTGTGACC3′), 11BHSD2 (forward: 5′TTTGGTGCACTTGAGCTGAC3′; reverse: 5′GGTATGGCCATGCTCTGTGCT3′), nephrin (forward: 5′ACTACGCCCTCTTCAAATGCA3′; reverse: 5′TCGAGGGCCTCATACCTGAT3′), G6PD (forward: 5′GTTAAATGGGCCAGCGAAG3′; reverse: 5′CCTGCTCTGATGGCAGG3′), and glyceraldehyde 3-phosphate dehydrogenase (forward: 5′TGTGTCCGTCGTGGATCTGA3′; reverse: 5′TTGCTGGTAAGTCGACGGAG3′).

**Histopathology quantification**

The kidneys of the mice were obtained and fixed with 4% paraformaldehyde overnight at room temperature before they were processed into paraffin. Thereafter, 4-μm sectioned samples were used for periodic acid-Schiff (PAS) staining and Sirius red staining. Tubular injury was assessed at 200× magnification in samples from each group that were stained with PAS. Tubular injury was assessed using 15 non-overlapping fields of view. Tubular injury was defined as the presence of tubular epithelial swelling, tubular epithelial vacuolization, tubular necrosis, and loss of brush border, and was categorized into 5 grades from 0 to 4, as described previously. Glomerulosclerosis index quantification was performed using 20 PAS-stained glomeruli. The glomeruli were scored on a 0–4 scale for glomerulosclerosis, as described previously. The scores for all glomeruli were averaged and presented as the glomerulosclerosis index. Tubular interstitial fibrosis quantification was performed using 20 Sirius red-stained sections at 200× magnification. The fibrosis area was quantified using ImageJ software (US National Institutes of Health, Bethesda, MD) and presented as the percentage area of fibrosis. Nephrin staining was performed on cryosections. Briefly, sectioned slides were incubated with an antibody against nephrin (Progen Biotechnik GmbH, Heidelberg, Germany) at 100× dilution overnight at 4°C. Nephrin expression was presented as the percentage of the entire glomerulus area that was positive for nephrin. Fifteen glomeruli were assessed for nephrin expression using the ImageJ software.
Assessment of reactive oxygen species production

The assessment of ROS production was performed by cryosections staining with dihydroethidium (DHE). Briefly, cryosections were incubated with 5-µM DHE in dimethyl sulfoxide at 37°C for 30 minutes. The quantification of DHE intensity was performed using 15 visual fields at 200× magnification.

Statistical analyses

All data are presented as mean ± SEM. We have assumed that the groups were not normally distributed. We therefore performed statistical analysis of multiple groups using the non-parametric Kruskal–Wallis test, followed by Mann–Whitney U-test between two groups. A P value of <0.05 was considered significant.

Results

Serum aldosterone and renin gene expression level

Serum aldosterone levels were significantly greater in Cry 1, 2 DKO mice compared with wild-type mice. High levels of serum aldosterone in Cry 1, 2 DKO mice were persistent even under high-salt treatment, whereas serum aldosterone levels in wild-type mice were significantly decreased following high-salt treatment (Figure 1A). To assess whether aldosterone production is independent of renin gene expression, we examined renin gene expression in the whole kidney of Cry 1, 2 DKO and wild-type mice. We noted that renin gene expression in the whole kidney was similar among the groups (Figure 1B). The aldosterone receptor (MR) expression in the whole kidney was also similar among the groups. Since MRs are nonspecific receptors and their specificity is determined by 11β hydroxysteroid dehydrogenase type 2 (11BHSD2), we measured 11BHSD2 expression in the whole kidney as well. However, we did not note any difference in 11BHSD2 expression among all groups (Figure 1C and 1D).

Blood pressure examination
Blood pressure was similar between the Cry 1, 2 DKO mice receiving normal salt treatment (CRYNS) and wild-type mice treated with normal salt (WTNS) groups. Under high-salt treatment, the systolic blood pressure in the Cry 1, 2 DKO mice receiving high-salt treatment (CRYHS) group increased significantly. Moreover, spironolactone treatment did not decrease the blood pressure in the Cry 1, 2 DKO mice receiving high-salt and spironolactone treatment (CRYSPI) group. However, hydralazine treatment significantly decreased the blood pressure in the Cry 1, 2 DKO mice receiving high-salt and hydralazine treatment (CRYHYD) group (Figure 2).

Blood and urine biochemical analysis

Creatinine clearance levels were similar between Cry 1, 2 DKO and wild-type mice receiving normal salt treatment (Figure 3A). High-salt treatment increased the creatinine clearance in Cry 1, 2 DKO mice but not in wild-type mice. Spironolactone or hydralazine treatment in high-salt-treated Cry 1, 2 DKO mice did not decrease the creatinine clearance. To further examine the effect of hyperaldosteronism on kidney function, we assessed the urinary albumin levels. Under normal salt treatment, urinary albumin levels were significantly higher in Cry 1, 2 DKO mice compared with wild-type mice (Figure 3B). High-salt treatment in Cry 1, 2 DKO mice further increased urinary albumin excretion. However, treatment with spironolactone, but not hydralazine, decreased urinary albumin excretion. Sodium and potassium concentration both in serum and urine were the same between Cry 1, 2 DKO and wild type mice under the same treatment. Treatment with high salt and spironolactone or hydralazine in Cry 1, 2 DKO and WT mice induced no statistically significant difference in serum sodium and potassium concentration. High salt treatment increased the urine sodium and potassium concentration both in Cry 1, 2 DKO and wild type mice. Treatment with high salt and spironolactone or hydralazine in Cry 1, 2 DKO mice also resulted in the increase of urine sodium and potassium concentration (Figure 3C-F).

Kidney histology assessment
Kidney tubular injury and kidney fibrosis were measured in the tubular area of the kidney by PAS staining and Sirius red staining respectively (Figure 4A). Glomerulosclerosis was measured in glomerular area of the kidney using PAS staining (Figure 4A). Kidney tubular injury was observed in the CRYNS group but not in the WTNS group (Figure 4B). The mice in the CRYHS group exhibited more severe kidney tubular injury compared with mice in the wild-type mice receiving high-salt treatment (WTHS) and CRYNS groups. Moreover, spironolactone treatment ameliorated kidney tubular injury in the CRYSPI group, whereas hydralazine treatment did not improve kidney tubular injury in the CRYHYD group. Examination of glomerulosclerosis and quantification of tubular interstitial fibrosis were similar among the treatment groups (Figure 4C and 4D). Nephrin expression in the glomerular area was analyzed by immunostaining (Figure 5A). Mice in the CRYNS group showed lower levels of nephrin expression compared to those in the WTNS group (Figure 5B and 5C). The level of nephrin expression in the CRYHS group was lower compared with those in the WTHS and CRYNS groups. However, treatment with spironolactone, but not hydralazine, improved the levels of nephrin expression in high-salt-treated Cry 1, 2 DKO mice.

Reactive oxygen species (ROS) production

Production of ROS in the kidney was examined using DHE staining depicted in figure 6A. We observed significantly higher levels of ROS in the CRYNS group compared to the WTNS group. Mice in the CRYHS group showed a further increase in ROS production in the kidney. Spironolactone treatment in high-salt-treated Cry 1, 2 DKO mice caused a decrease in ROS production, whereas hydralazine treatment did not (Figure 6B). To further examine the cause of the increased ROS production, we examined the expression of glucose-6-phosphate dehydrogenase (G6PD). The levels of G6PD expression decreased in the CRYNS and CRYHS groups compared to the WTNS and WTHS groups, respectively. Treatment with spironolactone, but not hydralazine, improved the levels of G6PD expression in high-salt-treated Cry 1, 2 DKO mice (Figure 6C).
Discussion

In the present study, we demonstrated chronic hyperaldosteronism pathological effect towards the kidney independent of high salt treatment and hypertension in the Cry 1, 2 DKO mice. Our model showed that even under normal salt treatment, chronic hyperaldosteronism resulted in albuminuria, tubular injury, decreased nephrin expression, and increased ROS production in the absence of hypertension. Notably, the pathological processes due to the hyperaldosteronism have taken place after a long follow-up period (32 weeks). We further confirmed that the high-salt exposure in the previous condition induced more severe kidney damage compared with that observed in hyperaldosteronism case alone. Finally, we also demonstrated that kidney damage induced by hyperaldosteronism and high-salt exposure is independent of blood pressure.

Despite the high plasma aldosterone levels, the Cry 1, 2 DKO mice under normal salt treatment showed no different of blood pressure and serum potassium level compared with those on wild type mice under the same treatment. Although hyperaldosteronism is frequently associated with hypertension and hypokalemia, normotensive or normokalemic patients with hyperaldosteronism have also been reported.\textsuperscript{20-22} Therefore, hypertension should not be used as a prerequisite for the diagnosis of primary aldosteronism.\textsuperscript{20} It is also suggested that normokalemia should not exclude the possibility of primary hyperaldosteronism.\textsuperscript{23}

We noted that the Cry 1, 2 DKO mice, in the present study, developed albuminuria following normal salt treatment and in the absence of elevated blood pressure. Aldosterone exerts its effect by binding with MRs. In the kidney, MRs are expressed mainly in the distal tubules and collecting ducts.\textsuperscript{24} A lower expression of MRs have been reported in glomeruli, particularly in mesangial cells\textsuperscript{25} and podocytes\textsuperscript{26} in which aldosterone may act directly. In unilateral nephrectomized rats receiving aldosterone and high-salt treatment, proteinuria was found to be associated with a decrease in the levels of nephrin, which is a podocyte-related protein.\textsuperscript{26} Nephrin is expressed specifically in the slit diaphragm of podocytes and
therefore plays an important role in the function of the glomerular filtration barrier. In the present study, we observed that Cry 1, 2 DKO mice showed a decrease in the levels of nephrin expression. Therefore, we suggest that a decrease in the levels of nephrin expression in Cry 1, 2 DKO mice results in the disruption of the glomerular filtration barrier, and thus influences, at least in part, the urinary albumin level.

In our study, Cry 1, 2 DKO mice characterized by hyperaldosteronism showed increased kidney ROS production in the absence of high-salt exposure, which is consistent with the findings of previous studies. Administration of the ROS detoxifier agent tempol to uninephrectomized rats receiving high-salt treatment and aldosterone has been reported to ameliorate kidney damage, indicating that ROS production might be one of the responsible factors for aldosterone-induced kidney damage. The results in the present study suggest that this applies to cases with only hyperaldosteronism as well. In addition, we noted that G6PD expression decreased in Cry 1, 2 DKO mice. G6PD is the first enzyme in the pentose phosphate pathway, which is an important source of NADPH (Nicotinamide Adenine Dinucleotide Phosphate). A previous study involving aldosterone- and salt-treated mice indicated a decrease in the expression and activity of G6PD in the aorta; this promotes increased ROS accumulation due to the failure in maintaining the production of NADPH that is an important intracellular reducing equivalent. G6PD deficiency in mice also reportedly results in increased renal oxidative stress and urinary albumin levels. Thus, the decrease in the expression levels of G6PD in Cry 1, 2 DKO mice may play a role in the increase in ROS production and urinary albumin levels.

Compared with animal models combining unilateral nephrectomy as well as high-salt and mineralocorticoid exposure, wherein a relatively short-term treatment (4–8 weeks) produced severe kidney damage including glomerulosclerosis and kidney fibrosis, the kidney damage observed in the normal salt-treated Cry 1, 2 DKO mice over 32 weeks was less severe. Moreover, even after 32 weeks of high-salt treatment, the Cry 1, 2 DKO mice did not develop glomerulosclerosis or interstitial fibrosis. Based on these observations, we cannot rule out the fact that chronic endogenous hyperaldosteronism
may have induced adaptive mechanisms, which could have limited the deleterious effects of aldosterone. These results may reflect clinical findings in primary aldosteronism patients who often present with microalbuminuria and increased glomerular filtration rate (GFR)\(^{31}\) that can be successfully treated either by adrenalectomy or MR blocker treatment.\(^{32-34}\) Moreover, severe kidney damage is believed to develop only after several years.\(^{35}\)

In the present study, we confirmed that aldosterone combined with high-salt treatment can induce severe kidney damage. High-salt exposure has been proved to exert pathological effects on the kidney. In rats with renal transplantation, an elevated salt intake (8% NaCl) induces albuminuria, glomerulosclerosis, and tubulointerstitial injury.\(^{36}\) In a rat model of metabolic syndrome, high-salt treatment for 4 weeks also promotes hypertension, proteinuria, and kidney damage.\(^{37}\) In a genetic rat model with lower nephron density that received high-salt treatment for 6 weeks, increased blood pressure and albuminuria were observed.\(^{38}\) Interestingly, the pathological effect of salt is related to MR activation. A previous study showed that MR blockers could ameliorate hypertension, proteinuria, and kidney injury in unilateral nephrectomized rats receiving 4 weeks of high-salt treatment.\(^{39}\) Another study in salt-sensitive Dahl rats receiving high-salt treatment indicated the presence of increased blood pressure, albuminuria, kidney damage, and impaired MR signaling in the kidney.\(^{40}\) Therefore, we suggest that kidney damage in high-salt-treated Cry 1, 2 DKO mice was caused by MR activation due to both hyperaldosteronism and high-salt exposure.

High-salt-induced kidney damage in Cry 1, 2 DKO mice was independent of blood pressure. We used low-dose spironolactone (an MR blocker) to inhibit the effect of aldosterone without decreasing blood pressure. It has been reported previously that the administration of spironolactone at a dose of 6-mg/kg body weight/day did not decrease blood pressure in an angiotensin II-treated mice model. Moreover this low dose of spironolactone did not decrease urinary potassium level as already observed.\(^{41}\) Spironolactone treatment decreased the urinary albumin level, nephrin expression, and ROS production but did not change the creatinine clearance in the CRYHS group. The increase in creatinine clearance in cases of
hyperaldosteronism and high-salt exposure as observed in Cry 1, 2 DKO mice may be a result of a functional adaptation, which is induced by increased sodium reabsorption and body fluid volume, and may lead to hypertension, an increase in renal perfusion pressure, suppression of renin activity, and a decrease in intrarenal vascular resistance. In WT mice, the creatinine clearance did not increase because both blood pressure and aldosterone levels remained stable on high salt diet. Since the dose of spironolactone used in the present study did not result in a decrease in the blood pressure in Cry 1, 2 DKO mice, we suggest that renal perfusion and intrarenal vascular resistance did not change as well, which would explain the stable creatinine clearance level. To further analyze the effect of aldosterone on the development of kidney damage independent of blood pressure, we administered a direct vasodilator, hydralazine. Hydralazine decreases blood pressure by inducing arterial smooth muscle relaxation. Therefore the effect in this group is representative of a decrease in blood pressure without any interference in the aldosterone-MR interaction. Despite the decrease in blood pressure, treatment with hydralazine in high-salt-treated Cry 1, 2 DKO mice did not improve creatinine clearance, albuminuria, nephrin expression, and ROS production. In another study of rats treated with aldosterone, 1% NaCl, and hydralazine for 4 weeks, a similar result was noted; in this study, hydralazine treatment decreased blood pressure but did not change the creatinine clearance value, albuminuria, kidney sclerosis, or ROS production. In certain normal, hypertensive, and congestive heart failure patients, hydralazine does not appear to influence GFR. The fact that the change in renal plasma flow produced by a vasodilator did not change the GFR indicates the presence of a filtration pressure disequilibrium. Therefore, given the fact that treatment with spironolactone, but not hydralazine, prevented kidney damage, we suggest that the deleterious effects of salt and hyperaldosteronism on the kidney were specifically due to MR activation.

Considering the role of cryptochromes on the circadian rhythm, the effect of cryptochrome deletion in Cry 1, 2 DKO mice cannot be disregarded. The increase in urinary albumin levels, decrease in nephrin expression, and increase in ROS production in high-salt-treated CRYHS mice were inhibited by
spironolactone treatment. Thus, we assumed that the kidney damage that developed in the Cry 1, 2 DKO mice was related to hyperaldosteronism.

Finally, the levels of nephrin expression and ROS production in the mice in the spironolactone-treated group were better than those in the CRYNS group and comparable to those in the WTNS group. Thus, in addition to the inhibition of the combined effect of hyperaldosteronism and salt, spironolactone may also suppress the effect of hyperaldosteronism alone.

In conclusion, our study demonstrated that the deleterious effects of aldosterone on the kidney might be independent of high-salt exposure and high blood pressure, even though high-salt treatment exacerbates these effects. Based on our findings, we suggest that controlling blood pressure may not be sufficient to protect the kidney in primary aldosteronism; however, the direct blocking of aldosterone may be sufficient to protect the kidney in such cases.

Disclosure

Authors declared no competing interest.

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**Figure Legends**

Figure 1: Serum aldosterone concentration (A) and renin (B), mineralocorticoid receptor (MR) (C), and 11 beta hydroxysteroid dehydrogenase type 2 (11BHSD2) (D) mRNA expression. Serum aldosterone concentration measurement was performed in 5–9 mice. (B, C, and D) The graphs represent the ratio between mRNA expression normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in the whole kidney (n = 4). Data are presented as mean ± SEM. *P < 0.05 using Mann–Whitney U test.
Figure 2: Systolic blood pressure measured in conscious mice using the tail-cuff method. Data are presented as mean ± SEM. n = 6–14. *P < 0.05 using Mann–Whitney U test.
Figure 3: Creatinine clearance (A), albuminuria (B), serum potassium level (C), serum sodium level (D), urine potassium level (E), urine sodium level (F) of 32 week treated mice. Data are presented as mean ± SEM. n = 6–13. *P < 0.05 using Mann–Whitney U test.

Figure 4: Representative pictures of periodic acid-Schiff (PAS) staining of the tubular area, Sirius red staining of tubular area and PAS staining of the glomerular area (A). The graphs represent the quantification of the tubular injury score (B), glomerulosclerosis (C), and tubular interstitial fibrosis (D). Tubular injury was presented by PAS staining of the tubular area as tubular vacuolization.
(arrow) and loss of brush border (arrowhead). Data are presented as mean ± SEM. n = 6–14. *P < 0.05 using Mann–Whitney U test.

**Figure 5A**

![WTNS WTHS CRYNS CRYHS CRYSPI CRYHYD](image)

**Figure 5B**

![Bar chart](image)

**Figure 5C**

![Bar chart](image)

**Figure 5: Nephrin expression.** (A) A representative image of nephrin immunofluorescent staining and (B) quantification of the signal as the percentage of the glomerulus area (n = 5–10). (C) The graphs represent the ratio between mRNA expression normalized to the expression of GAPDH mRNA (n = 5–7). Data are presented as mean ± SEM. *P < 0.05 using Mann–Whitney U test.

**Figure 6A**

![WTNS WTHS CRYNS CRYHS CRYSPI CRYHYD](image)
Figure 6: Dihydroethidium (DHE) staining (A), quantification of DHE staining (B), and glucose-6-phosphate dehydrogenase (G6PD) mRNA expression (C). DHE staining quantification was performed in 5 samples in each group. (A) The DHE positive staining showed as red fluorescent staining on the cell nucleus on glomerular (G) and tubular (T) area. (B) Quantification of DHE staining intensity. (C) The graphs represent the ratio between G6PD mRNA expression normalized to the expression of GAPDH mRNA (n = 6–11). Data are presented as mean ± SEM. *P < 0.05 using Mann–Whitney U test.