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ORIGINAL ARTICLE

# Iron restriction inhibits renal injury in aldosterone/salt-induced hypertensive mice

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**Excess iron is associated with the pathogenesis of several renal diseases. Aldosterone is reported to have deleterious effects on the kidney, but there have been no reports of the role of iron in aldosterone/salt-induced renal injury. Therefore, we investigated the effects of dietary iron restriction on the development of hypertension and renal injury in aldosterone/salt-induced hypertensive mice. Ten-week-old male C57BL/6J mice were uninephrectomized and infused with aldosterone for four weeks. These were divided into two groups: one fed a high-salt diet (Aldo) and the other fed a high-salt with iron-restricted diet (Aldo-IR). Vehicle-infused mice without a uninephrectomy were also divided into two groups: one fed a normal diet (control) and the other fed an iron-restricted diet (IR) for 4 weeks. As compared with control and IR mice, Aldo mice showed an increase in both systolic blood pressure and urinary albumin/creatinine ratio, but these increases were reduced in the Aldo-IR group. In addition, renal histology revealed that Aldo mice exhibited glomerulosclerosis and tubulointerstitial fibrosis, whereas these changes were attenuated in Aldo-IR mice. Expression of intracellular iron transport protein transferrin receptor 1 was increased in the renal tubules of Aldo mice compared with control mice. Dietary iron restriction attenuated the development of hypertension and renal injury in aldosterone/salt-induced hypertensive mice.**
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**Keywords:** aldosterone; iron; renal injury; salt; transferrin receptor 1

## INTRODUCTION

It is known that aldosterone has an important role in regulating electrolyte and fluid homeostasis through the activation of mineralocorticoid receptor (MR) in the kidney. Several studies have shown that in addition to aldosterone's classic effects on renal electrolyte and fluid homeostasis, direct MR activation by aldosterone is reported to lead to renal injury in patients with hypertension and hypertensive experimental models.[1–5] For instance, Blasi *et al.* have shown that chronic administration of aldosterone and salt induce inflammation and fibrosis in the kidney and treatment with a selective MR antagonist, eplerenone, attenuates the aldosterone-induced renal injury in rats.[1] These reports suggest that aldosterone has deleterious effects on the kidney. However, the precise mechanisms of aldosterone-induced renal injury are largely unknown.

Iron is an essential element for maintaining physiological function in the body, while excess iron leads to tissue damage by oxidative stress through the Fenton/Haber–Weiss reaction.[6] Iron accumulation is observed in the atherosclerotic lesions of apolipoprotein E-deficient mice and in the proximal tubules of human chronic renal disease.[7,8] Furthermore, we have recently shown that renal iron accumulation and superoxide production are observed in a chronic kidney disease (CKD) model in rats.[9] Thus, iron is considered to be involved in the pathophysiology of several cardiovascular and renal diseases. Of interest, we have previously reported that dietary iron restriction prevents the development of hypertension and proteinuria in rats with CKD.[9] In that study, renal mineralocorticoid receptor (MR) expression in the nuclear fraction was increased in CKD rats as compared with the controls, whereas circulating aldosterone levels were decreased in CKD rats compared with the controls. In general, it is widely accepted that exogenous high aldosterone and salt induce renal injury.[1] In addition, patients with primary aldosteronism (PA) frequently complicates renal dysfunction,[10,11] and high plasma aldosterone levels are at a high risk of renal disease.[12] Thus, in the present study, we aimed to assess the effects of dietary iron restriction on renal injury directly induced by exogenous high aldosterone and salt in mice.

## METHODS

### Animals

Eight-week-old male C57BL/6J mice (20–23 g) were used for this study. All the mice were individually housed and were maintained on a 12-h light/dark cycle and had free access to food and water. All our experimental procedures were approved by the Animal Research Committee of Hyogo College of Medicine. After a 1-week training period for the measurements of blood pressure, the mice had a left uninephrectomy, carried out under pentobarbital sodium anesthesia (50 mg kg$^{-1}$, intraperitoneal injection). After that, an osmotic

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minipump (Alzet, Cupertino, CA, USA) was implanted subcutaneously to infuse vehicle or aldosterone (0.15 μg $h^{-1}$, Sigma-Aldrich, St Louis, MO, USA) as previously described.[13,14] Aldosterone-infused mice were divided into two groups: one fed a high-salt diet (8% NaCl; Aldo, $n=8$) and the other fed a high-salt with iron-restricted diet (Aldo-IR, $n=8$) for 4 weeks. Vehicle-infused mice without a uninephrectomy were also divided into two groups: one fed a normal diet (control, $n=6$) and the other fed an iron-restricted diet (IR, $n=4$) for 4 weeks. The nutrients of the normal diet consisted of 33% of cornstarch, 22% of casein, 5% of cellulose, 30% of sucrose, 5% corn oil, 4% of mineral mixture and 1% of vitamin mix. The mineral mixture contained: 0.43% of $CaHPO_4 \cdot 2H_2O$, 34.31% of $KH_2PO_4$, 25.06% of NaCl, 0.623% of $FeC_6H_5O_7 \cdot 5H_2O$, 4.8764% of $MgSO_4$, 0.02% of $ZnCl_2$, 0.121% of $MnSO_4 \cdot 5H_2O$, 0.156% of $CuSO_4 \cdot 5H_2O$, 0.0005% of KI, 29.29% of $CaCO_3$, 0.0025% of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 5.11% of microcrystal line cellulose. The high-salt diet was composed of a normal diet to which NaCl was added to achieve 8% of the total content by weight. The iron-restricted diet was based on the normal diet, but with a mineral mixture free of $FeC_6H_5O_7 \cdot 5H_2O$ as previously described.[15]

In a separate study, 10-week-old uninephrectomized Aldo mice with high-salt diet were divided into two groups: the first given plain drinking water (Aldo, $n=4$) and the second given hydralazine (50 mg $kg^{-1}$ per day in drinking water; Aldo-Hyd, $n=4$) for 4 weeks. Systolic blood pressure (SBP) was measured by a noninvasive computerized tail-cuff system every 2 weeks (Muromachi Kikai, Tokyo, Japan). Twenty-four-hour urine samples were collected in the metabolic cage after 28 days of aldosterone infusion. At the end of the study, blood samples were obtained from the heart, and the kidney and liver were removed and weighed. Coronal sections of the kidney were fixed in buffered 4% paraformaldehyde, followed by embedding in paraffin for histological evaluation. The remainders of kidney and the liver were snap-frozen in liquid nitrogen.

### Biochemical measurements

Urinary concentrations of albumin, creatinine and 8-hydroxy-2′-deoxyguanosine (8-OHdG) were measured by ELISA, enzymatic assay and enzyme immunoassay, respectively. Serum and renal iron concentrations were determined by atomic absorption spectrometry. Peripheral blood cell count was measured using an automatic cell count analyzer (Horiba, Kyoto, Japan).

### Western blot analysis

The total protein homogenate (30 μg) from the kidney was separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The expression levels of proteins were detected by an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL, USA). The antibodies used were against mouse anti-transferrin receptor 1 (TfR1; dilution 1:1000; Zymed Laboratories, South San Francisco, CA, USA) and rabbit anti-glyceraldhyde-3-phosphate dehydrogenase (dilution 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA). Expression of TfR1 was standardized on the basis of glyceraldhyde-3-phosphate dehydrogenase expression.

### Histological analysis

Kidney tissues were quickly fixed with buffered 4% paraformaldehyde, embedded in paraffin and cut into 4-μm-thick sections. Periodic acid–Schiff and Masson's trichrome staining were performed using serial sections. Glomerulosclerotic lesions were evaluated by semiquantitative scoring using the method as previously described.[16] Tubulointerstitial fibrosis areas were semiquantified using image J software and expressed as a percentage of the total area.

### Immunohistochemical analysis

Kidney tissues were immunohistochemically stained with a primary mouse anti-TfR1 antibody (dilution 1:50; Zymed Laboratories) and mouse anti-8-OHdG antibody (dilution 1:1000; JaICa, Shizuoka, Japan). Immunostains were visualized with the use of an avidin-biotin-peroxidase conjugate and 3,3′-diaminobenzidine substrate. Every section was counterstained with hematoxylin.

### Gene expression analysis

Total RNA was isolated from the tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), as previously described.[17,18] Real-time reverse transcription-PCR assays were performed using the ABI PRISM 7900 with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems, Alameda, CA, USA). Real-time reverse transcription-PCR assays were performed using mouse specific primers for ferritin heavy chain 1 (Fth1; Mm0085070_g1), ferritin light chain 1 (Ftl1; Mm03030144_g1), and serum and glucocorticoid-regulated protein kinase 1 (SGK1; Mm00441387_g1). Glyceraldhyde-3-phosphate dehydrogenase (Mm99999915_g1) was used as internal control.

### Statistical analysis

Data are expressed as means ± s.e.m. Statistical analysis was performed using one-way analysis of variance. Analysis of variance (Kruskal–Wallis test, followed by Mann–Whitney *U*-test) was used for statistical comparisons. Probability values less than 0.05 were considered statistically significant.

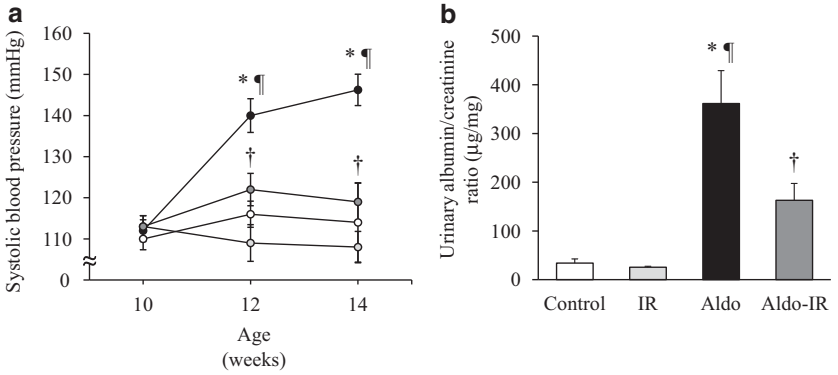


**Figure 1** Iron restriction inhibited the development of hypertension and albuminuria in aldosterone/salt-induced hypertensive mice. (**a**) The profile of systolic blood pressure in control (white circle), IR (light gray circle), Aldo (black circle), and Aldo-IR (gray circle) mice. (**b**) Urinary albumin/creatinine ratio in control (white bar), IR (light gray bar), Aldo (black bar) and Aldo-IR (gray bar) mice. Control, mice fed a normal salt diet and vehicle infusion; IR, mice fed an iron-restricted diet and vehicle infusion; Aldo, uninephrectomized mice fed a high-salt diet and aldosterone infusion; Aldo-IR, uninephrectomized mice fed a high-salt with iron-restricted diet and aldosterone infusion. *$P<0.05$ compared with control mice, ¶$P<0.05$ compared with IR mice, †$P<0.05$ compared with Aldo mice.

## RESULTS

### Physiological and hematological parameters in aldosterone/salt-induced hypertensive mice

SBP in IR mice was not different from that in control mice. Aldo mice showed an increase of SBP compared with control mice, whereas it was attenuated in Aldo-IR mice (Figure 1a). As shown in Table 1, body weight was decreased in IR and Aldo-IR mice compared with control mice. The kidney weight to body weight ratio was not different between control and IR mice, and that ratio was increased in Aldo and Aldo-IR mice compared with control mice. Serum iron concentrations were decreased in Aldo-IR mice compared with the other groups, whereas blood hemoglobin and hematocrit levels did not differ among these groups. MCV and MCH were decreased in IR, Aldo and Aldo-IR mice compared with control mice. Survival rates were not different among four groups, because all of the mice survived throughout the experimental period.

**Table 1 Physiological and hematological parameters in aldosterone/salt-induced hypertensive mice**

| | *Control* | *IR* | *Aldo* | *Aldo-IR* |
|---|---|---|---|---|
| Body weight (g) | 29.3±1.0 | 25.4±0.6[a] | 27.9±0.5 | 24.6±0.4[a] |
| Kidney weight/body weight ratio (mg g$^{-1}$) | 5.3±0.3 | 5.8±0.2 | 9.5±0.2[a,b] | 11.0±0.7[a,b,c] |
| Serum iron concentrations (μg dl$^{-1}$) | 151.5±7.4 | 141.0±6.6 | 154.8±7.9 | 118.7±5.1[a,c] |
| Hemoglobin (g dl$^{-1}$) | 14.5±0.3 | 13.7±0.1 | 13.7±0.1 | 13.0±1.5 |
| Hematocrit (%) | 51.3±1.2 | 47.0±0.41 | 47.6±1.2 | 43.0±5.1 |
| MCV (μm$^3$) | 53±1 | 47±1[a] | 49±0[a] | 44±1[a,c] |
| MCH (pg) | 14.8±0.0 | 14.1±0.1[a] | 13.9±0.1[a] | 13.0±0.2[a,b,c] |

Abbreviations: Aldo, uninephrectomized mice fed a high-salt diet and aldosterone infusion; Aldo-IR, uninephrectomized mice fed a high-salt with iron-restricted diet and aldosterone infusion; Control, mice fed a normal salt diet and vehicle infusion; IR, mice fed an iron-restricted diet and vehicle infusion; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume.
Values are means±s.e.m.
[a]$P<0.05$ compared with control mice.
[b]$P<0.05$ compared with IR mice.
[c]$P<0.05$ compared with Aldo mice.

### Effects of iron restriction on albuminuria and renal structure in aldosterone/salt-induced hypertensive mice

Urinary albumin/creatinine ratio was not different between control and IR mice. Aldo mice exhibited higher urinary albumin/creatinine ratio than control mice, whereas dietary iron restriction suppressed the increase of this ratio in Aldo mice (Figure 1b). Glomerulosclerosis and tubulointerstitial fibrosis were not mostly detected in control and IR mice (Figures 2a–d). Aldo mice exhibited glomerulosclerosis, whereas this change was attenuated in Aldo-IR mice (Figures 2a and c). In addition, Aldo mice showed increased tubulointerstitial fibrosis consistent with the previous report,[19] whereas it was suppressed in Aldo-IR mice (Figures 2b and d).

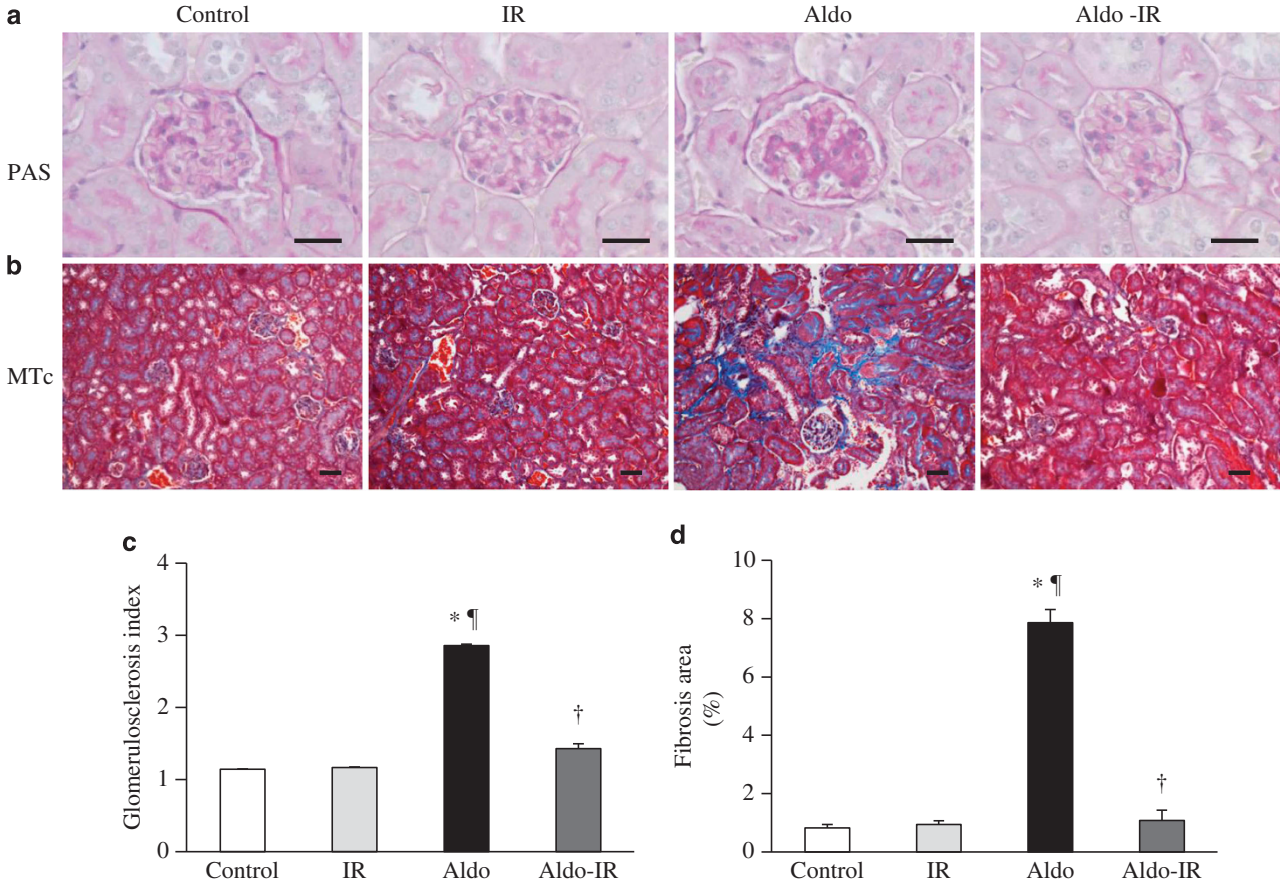


**Figure 2** Iron restriction attenuated glomerulosclerosis and tubulointerstitial fibrosis in aldosterone/salt-induced hypertensive mice. Representative images of (**a**) periodic acid–Schiff (PAS) and (**b**) Masson's trichrome (MTc) staining of the kidney sections. Scale bars: 200 μm. Semiquantitative analysis of (**c**) glomerulosclerosis index and (**d**) tubulointerstitial fibrosis. *$P<0.05$ compared with control mice, ¶$P<0.05$ compared with IR mice, †$P<0.05$ compared with Aldo mice.

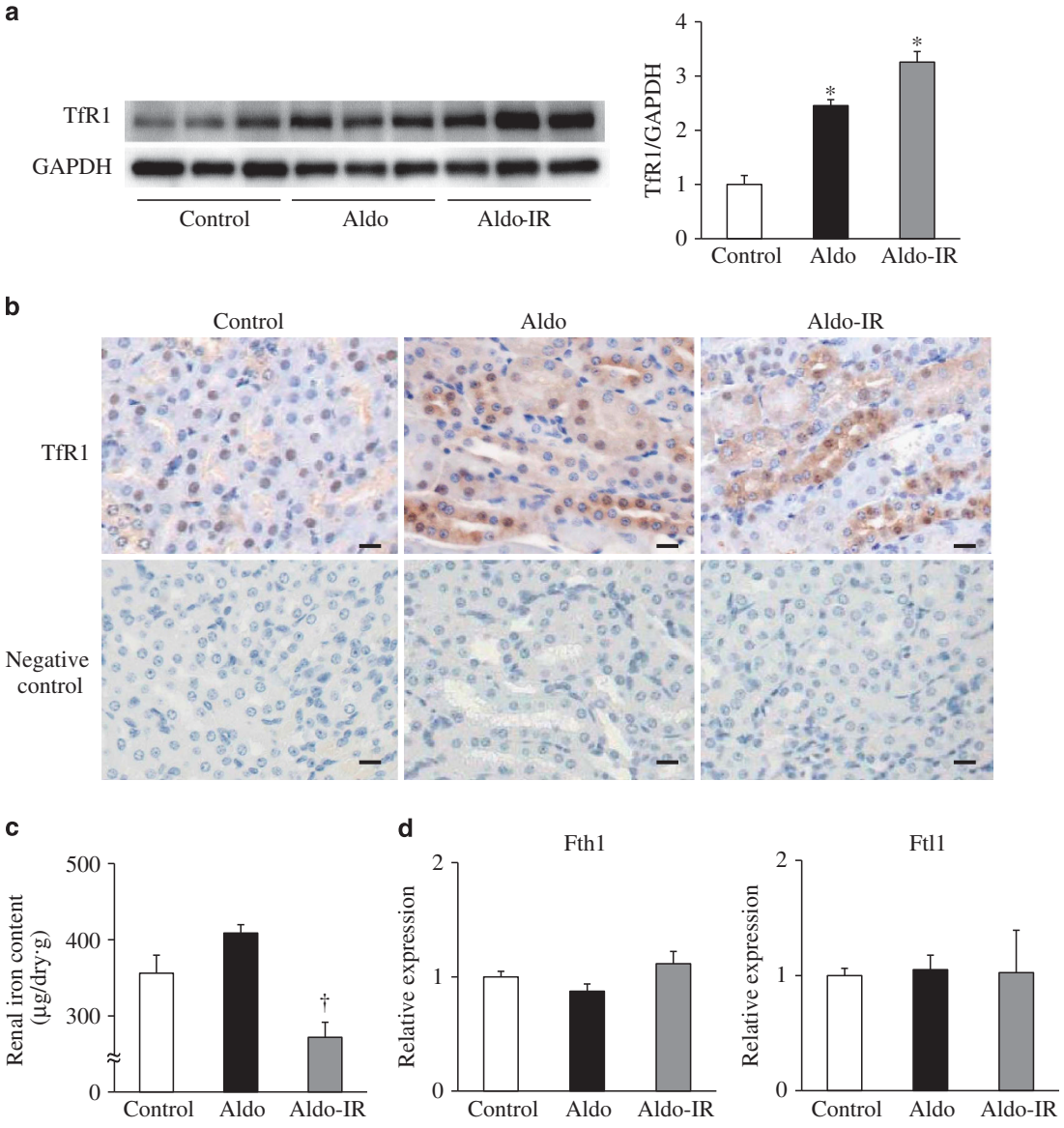


**Figure 3** Renal TfR1 expression was increased in aldosterone/salt-induced hypertensive mice. (**a**) Representative immunoblot and densitometric ratios of TfR1 in renal tissue homogenates. (**b**) Representative immunohistochemistry for TfR1 of the kidney sections. (**c**) Renal iron content. (**d**) Renal gene expression of Fth1 and Ftl1. Scale bar: 100 μm. $^{*}P<0.05$ compared with control mice, $^{\dagger}P<0.05$ compared with Aldo mice. GAPDH, glyceraldhyde-3-phosphate dehydrogenase.

**Effects of iron restriction on renal iron transporter and oxidative stress in aldosterone/salt-induced hypertensive mice**

To investigate whether iron transport is associated with the mechanism of aldosterone/salt-induced renal injury, we examined renal expression of TfR1, intracellular iron transport protein, in control, Aldo and Aldo-IR mice. Renal TfR1 expression levels were increased in both Aldo and Aldo-IR mice compared with control mice (Figure 3a). Immunohistochemical analysis showed that TfR1 was expressed in renal cortical and medullary tubules in the mouse kidney, and TfR1-positive cells were highly expressed in the renomedullary tubules of Aldo and Aldo-IR mice (Figure 3b). Renal iron content tended to be increased in Aldo mice compared with control mice, whereas it was decreased in Aldo-IR mice compared with Aldo mice (Figure 3c). To evaluate the ferritin state in these mice, we next assessed renal gene expression of Fth1 and Ftl1. Renal gene expression of Fth1 and Ftl1 was not different among three groups (Figure 3d). Also, hepatic gene expression of Fth1 and Ftl1 was not different among three groups (control, Aldo and Aldo-IR: Fth1; $1.0\pm0.2$, $0.8\pm0.1$ and $1.4\pm0.3$, Ftl1; $1.0\pm0.2$, $1.4\pm0.4$ and $1.7\pm0.2$, respectively).

We next examined renal expression of 8-OHdG, an oxidative stress marker, in aldosterone/salt-induced hypertensive mice. Immunohistochemical analysis demonstrated that 8-OHdG was expressed in the nuclei of renal tubules (both cortex and medulla) and its positive area increased in Aldo mice compared with control mice. However, 8-OHdG-positive area decreased in Aldo-IR mice (Figures 4a and b). Moreover, urinary 8-OHdG/creatinine ratio increased in Aldo mice compared with control mice, whereas it was suppressed in Aldo-IR mice (Figure 4c).

In addition, we examined renal SGK1 gene expression in order to assess aldosterone signaling pathway in these mice. Renal SGK1 gene expression levels were not different among three groups (control, Aldo and Aldo-IR: $1.0\pm0.2$, $0.6\pm0.1$ and $0.8\pm0.1$, respectively).

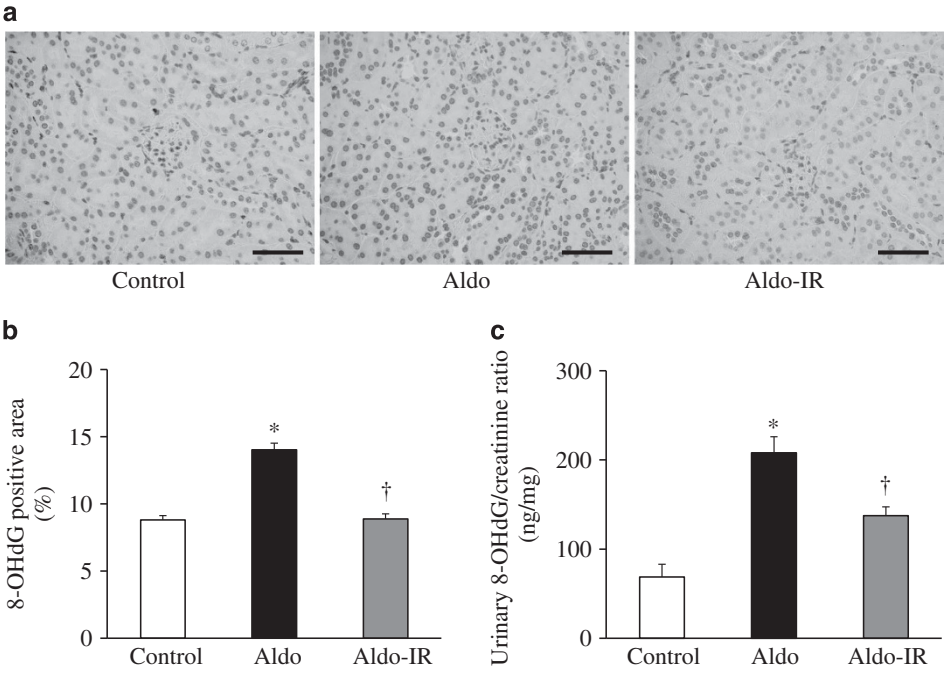


**Figure 4** Iron restriction reduced oxidative stress in aldosterone/salt-induced hypertensive mice. (**a**) Representative immunohistochemistry for 8-hydroxy-2′-deoxyguanosine (8-OHdG) of the kidney sections. Scale bar: 100 μm. (**b**) Semiquantitative analysis of 8-OHdG-positive area and (**c**) urinary 8-OHdG/creatinine ratio. $^{*}P<0.05$ compared with Control mice, $^{\dagger}P<0.05$ compared with Aldo mice. A full color version of this figure is available at the *Hypertension Research* journal online.

**Table 2 Effects of hydralazine in aldosterone/salt-induced hypertensive mice**

| | *Aldo* | *Aldo-IR* | *Aldo-Hyd* |
|---|---|---|---|
| Systolic blood pressure (mm Hg) | 146.3 ± 2.3 | 116.9 ± 3.1[a] | 100.5 ± 1.3[a,b] |
| Urinary albumin/creatinine ratio (μg mg$^{-1}$) | 361.5 ± 67.7 | 162.9 ± 34.6[a] | 263.9 ± 48.8[b] |
| Urinary 8-OHdG/creatinine ratio (ng mg$^{-1}$) | 207.9 ± 36.2 | 137.4 ± 22.4[a] | 229.9 ± 6.5[b] |

Abbreviations: Aldo, uninephrectomized mice fed a high-salt diet and aldosterone infusion; Aldo-IR, uninephrectomized mice fed a high-salt with iron-restricted diet and aldosterone infusion; Aldo-Hyd, uninephrectomized mice fed a high-salt diet and aldosterone infusion with hydralazine treatment.
Values are means ± s.e.m.
[a]$P<0.05$ compared with Aldo mice.
[b]$P<0.05$ compared with Aldo-IR mice.

**Effects of iron restriction on renal injury are independent of blood pressure reduction in aldosterone/salt-induced hypertensive mice**

Dietary iron restriction inhibited the development of hypertension and renal injury in Aldo mice. To investigate whether the effects of dietary iron restriction against aldosterone/salt-induced renal injury were dependent on blood pressure reduction, we next assessed urinary albumin/creatinine and 8-OHdG/creatinine ratios in Aldo mice when their blood pressure was lowered with a vasodilator, hydralazine. As shown in Table 2, hydralazine reduced SBP in Aldo mice to be lower than in Aldo-IR mice after 4 weeks treatment; however, urinary albumin/creatinine and 8-OHdG/creatinine ratios were not reduced in Aldo-Hyd mice compared with Aldo-IR mice. These results indicate that the effects of dietary iron restriction on renal injury are independent of blood pressure reduction in aldosterone/salt-induced hypertensive mice.

## DISCUSSION

The present study demonstrated that dietary iron restriction inhibited the development of hypertension and renal injury in aldosterone/salt-induced hypertensive mice. We also demonstrated that dietary iron restriction attenuated the aldosterone/salt-induced oxidative stress. Therefore, the beneficial effects of dietary iron restriction are considered to be exerted through inhibition of oxidative stress in renal injury of aldosterone/salt-induced hypertensive mice.

Iron is a necessary element in the body. However, excess iron promotes reactive oxygen species production through the Fenton/Haber–Weiss reaction, thereby leading to tissue damage. Therefore, iron is associated with the pathophysiology of various diseases. Nankivell *et al.* have reported that iron is accumulated in the renal tubules of human chronic renal disease.[8] Also, we have previously shown that iron accumulation and TfR1 expression are increased in the renal tubules of rats with CKD.[9] Generally, iron is transported into cells through TfR1. TfR1 expression is normally upregulated under low-iron conditions. By contrast, in high-iron conditions, TfR1 downregulated.[20] Wareing *et al.* have shown that serum iron concentration is involved in the proportion of renal iron filtration and reabsorption.[21] In this study, renal TfR1 expression was upregulated in Aldo-IR mice compared with control mice. As serum iron levels and renal iron content were lower in Aldo-IR mice than control mice, the low-iron condition was considered to increase renal TfR1 expression in Aldo-IR mice. On the other hand, serum iron levels were not different between control and Aldo mice. However, increased renal TfR1 expression was observed in Aldo mice compared with control mice. Furthermore, renal iron content tended to increase in Aldo mice compared with control mice. These results indicate that chronic administration of aldosterone and salt induces dysregulation of renal TfR1 expression, which may lead to renal iron reabsorption and induce renal injury in aldosterone/salt-induced hypertensive mice.

However, how renal TfR1 expression is regulated in Aldo mice remains unknown. Further studies are needed to explore this issue.

Chronic administration of aldosterone and salt induced renal injury, hypertension and oxidative stress in our experiment, consistent with the previous reports.[1,2] Meanwhile, hydralazine could not attenuate renal injury and oxidative stress in aldosterone/salt-administrated mice, despite reducing blood pressure in our experiment. Previous studies have reported that administration of aldosterone and salt induce renal injury and hypertension through oxidative stress.[2,14,20] These results suggest that oxidative stress is strongly involved in the mechanism of aldosterone/salt-induced renal injury and hypertension. In the present study, dietary iron restriction attenuated oxidative stress along with suppression of renal injury and hypertension. Thus, inhibitory effects of dietary iron restriction on hypertension may be exerted through inhibition of oxidative stress in aldosterone/salt-induced hypertensive mice.

Renal SGK1 gene expression was not different among the groups in our experiment. It is widely accepted that aldosterone modulates renal SGK1 expression through MR. In fact, there are some reports that renal SGK1 expression was increased in aldosterone/salt-induced renal injury model.[22–24] However, in their experiments, aldosterone dosage was $0.75\,\mu g\,h^{-1}$, which was five times higher than our experiment. Although we could not observe increased renal SGK1 gene expression in Aldo mice, the difference in these results may be caused by the difference in aldosterone dosage.

Clinically, PA frequently complicates renal dysfunction. Several studies have shown that urinary protein excretion is increased in patients with PA compared with patients with essential hypertension.[11,12] Proteinuria is one of the risk factors for the development of CKD.[25] In the present study, dietary iron restriction attenuates the development of albuminuria in aldosterone/salt-induced hypertensive mice. Since there is no report investigating the involvement of iron in the pathophysiology of PA, it might be necessary to consider the role of iron in patients with PA.

In conclusion, we found, for the first time, that dietary iron restriction inhibited the development of hypertension and renal injury in aldosterone/salt-induced hypertensive mice.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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