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ORIGINAL ARTICLE

Aliskiren reduced renal fibrosis in mice with chronic ischemic kidney injury—beyond the direct renin inhibition

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Chronic renal ischemia leads to renal fibrosis and atrophy. Activation of the renin-angiotensin–aldosterone system is one of the main mechanisms driving chronic renal ischemic injury. The aim of the present study was to define the effect of aliskiren in chronic ischemia of the kidney. Two-kidney, one-clip mice were used to study chronic renal ischemia. Aliskiren significantly lowered the blood pressure in mice with renal artery constriction (92.1 ± 1.1 *vs.* 81.0 ± 1.8 mm Hg, $P<0.05$). Renin expression was significantly increased in ischemic kidneys when treated with aliskiren. In addition, (Pro)renin receptor expression was decreased by aliskiren in ischemic kidneys. Aliskiren treatment significantly increased klotho expression and reduced the expression of fibrogenic cystokines, caspase-3 and Bax in ischemic kidneys. Histological examination revealed that aliskiren significantly reduced the nephrosclerosis score (4.5 ± 1.9 *vs.* 7.3 ± 0.4, $P<0.05$). Immunofluorescence staining also showed that aliskiren decreased the deposition of interstitial collagen I in ischemic kidneys. In conclusion, direct renin inhibition significantly reduced renal fibrosis and apoptosis following chronic renal ischemia.
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Keywords: chronic renal ischemia; direct renin inhibitor; renal fibrosis; renin-angiotensin–aldosterone system

INTRODUCTION

Preventing the progression of chronic kidney disease (CKD) is vital to ablate the increasing trend of end-stage renal disease.[1] To do so, determining the factors mediating the progression of CKD is the key.[2,3] Chronic ischemic nephropathy is thought to be one of the major determining factors in progression of CKD.[4] Approximately, 15% of end-stage renal disease is attributed to chronic ischemic nephropathy from renovascular disease.[4–6]

Activation of the renin-angiotensin–aldosterone system (RAAS) is one of the main mechanisms of chronic ischemic nephropathy-associated progressive of CKD.[7,8] RAAS activation is considered to have important roles in the renal fibrosis.[9] Renal ischemia stimulates the release of renin via afferent arteriole baroreceptors, or by sensing a decreased sodium chloride load to the distal nephron.[10] The subsequent production of angiotensin II induces vasoconstriction of both afferent and efferent arterioles, which results in an increase in glomerular pressure and glomerular filtration rate, and in further renal injury.[11] These hemodynamic changes occur immediately after ischemia. However, the RAAS alteration in chronic kidney ischemia is less understood.

Increased RAAS activity can act as a multifunctional cytokine such as a growth factor, a profibrogenic cytokine, and can display proinflammatory properties.[12,13] Renin itself has been identified as a mediator of profibrotic effects via binding to specific receptors.[14] These non-hemodynamic effects also have a role in renal kidney damage during chronic renal ischemia.

Inhibition of the RAAS is currently one of the most powerful therapies to slow the progression of CKD.[14,15] The angiotensin converting enzyme inhibitor and angiotensin receptor blocker are agents that block the activated RAAS. However, the effectiveness of these treatments is limited due to compensatory increases in plasma renin levels that lead to adjustments in angiotensin production and conversion.[16] A direct renin inhibitor, aliskiren, is now available to inhibit angiotensin production directly at its rate-limiting step.[17]

The goal of the present study was to understand the effect of aliskiren—a direct renin inhibitor—on renal fibrosis and possible mechanisms other than RAAS activation. To address this, we performed an animal study using the two-kidney, one-clip Goldblatt model for chronic ischemic nephropathy.[18]

METHODS

Animal model

Four-week-old female B6 mice (National Laboratory Animal Center, Taiwan) were assigned randomly into normal control ($n=8$) and experimental ($n=16$) groups. The experimental group received renal artery ligation surgery[19] and the control mice received sham surgery. Mice in the experimental group were assigned randomly into two groups: aliskiren treatment group ($n=8$) and

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non-treatment group (n=8). Mice of the treatment group received 25 mg kg^{-1} per day of aliskiren (Novartis, East Hanover, NJ, USA) via osmotic mini-pumps during renal artery ligation surgery, whereas the non-treatment group received no medical treatment for 3 weeks. The dose of aliskiren was determined according to previous studies.[20,21] Renal artery constriction (RAC) was performed by making dorsal, longitudinal incisions to expose the kidneys. The trunk of the left renal artery, which showed visible renal ischemia, was constricted by a vascular clamp (0.4–1.0 mm; S&T Fine Science Tools, North Vancouver, B.C. Canada); the right kidney remained intact. The kidney tissues were harvested 3 weeks after surgery and the kidney weight was measured. The blood pressure of study mice was determined before surgery and 3 weeks post surgery. The mean arterial pressure (2/3 diastolic pressure+1/3 systolic pressure) was determined before RAC and 3 weeks following RAC, with tail-cuff plethysmography.[22] All animals were given a normal diet (sodium, 0.25%; potassium, 0.76%) and tap water. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and was approved by the local ethics committee of Chang Gung Memorial Hospital.

Quantitative real-time PCR

Whole kidney extracts (10 mg) were used to isolate total RNA using a commercial kit (RNeasy Kit, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, including DNase treatment. Total RNA (5 μg) was then reverse transcribed by reverse transcriptase (Bio-Rad, Hercules, CA, USA) with random primers. Real-time PCR was performed in 25 μl SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) containing 0.6 mol l^{-1} primers and 1 μg cDNA, using an iQ5 real-time PCR detection system (Bio-Rad). The thermal cycling program and PCR primers are listed in Supplementary Table 1. Each PCR reaction was performed in triplicates, and the mean C_t value was used for static analysis. Messenger RNA expression was standardized to β-actin expression levels, followed by normalization to the control group.

Western blotting

Kidney tissues were homogenized and total protein was extracted using a commercial kit as per the manufacturer's instructions (Protein Extraction Kit, Millipore, Billerica, MA, USA). Kidney extracts (30 μg protein per lane) were mixed with a sample loading buffer and separated on 12% SDS-polyacrylamide gel. Proteins were electrotransferred on polyvinylidene fluoride membranes (0.2 μm: Immun-Blot, Bio-Rad). The antibodies used for western blotting are listed in the Supplementary Table 2. The intensity of each band was quantified using the NIH Image software, and the densitometric intensity corresponding to each band was normalized against β-actin expression.

Histopathological examination

Sections of paraffin-embedded specimens were stained with the periodic acid-Schiff reagents and counter-stained with hematoxylin to assess the degree of renal sclerosis. Four random and non-overlapping sections from each kidney tissue were examined under high power field (×200) by a pathologist blinded to the study. The nephrosclerosis scores were calculated by adding the scores of glomerulosclerosis, interstitial fibrosis and arterial sclerosis. The severity was graded from 0 to 3+ in each specimen.[23,24]

TUNNEL and immunofluorescence staining

Cryostat sections of renal tissue were used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNNEL) and immunofluorescence staining. TUNNEL staining was performed using a MEBSTAIN Apoptosis Kit Direct (MBL International, Woburn, MA, USA) according to the manufacturer's protocol. Sections of renal tissue were incubated with a primary antibody against mouse collagen I at 1:100 dilution, followed by incubation with a fluorescein isothiocyanate-conjugated anti-IgG antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Collagen I expression was determined by assigning an arbitrary grading scale (0 to 5+), based on the kidney interstitial staining intensity. The relative amount of staining was scored semiquantitatively from 0 to 5 as follows: (0) no staining; (1) trace staining; (2) slight staining; (3) moderate staining; (4) intensive staining; and (5) most intense staining. The nucleus was stained with Hoechst stain.

Statistical analysis

All data were expressed as mean ± s.e. The results of PCR and western blotting were plotted as the relative fold, as compared with the control group. Data from different study groups were compared using the Wilcoxon–Mann–Whitney test, and P-values of <0.05 were considered statistically significant.

RESULTS

Blood pressure and kidney size

The mean arterial pressure among control and experimental mice was not significantly different at the beginning of the study. Control mice did not exhibit changes in mean arterial pressure during the study period (before: 92.0 ± 1.8 *vs.* after: 92.2 ± 2.1 mm Hg). The mean arterial pressure increased significantly in mice with RAC (control: 91.3 ± 1.3 *vs.* RAC+ & aliskiren−: 98.9 ± 1.4 mm Hg, $P<0.05$). In mice with RAC, the mean arterial pressure decreased significantly after being treated with aliskiren (RAC+ and aliskiren−: 92.1 ± 1.1 *vs.* RAC+ and aliskiren+: 81.0 ± 1.8 mm Hg, $P<0.05$; Figure 1a).

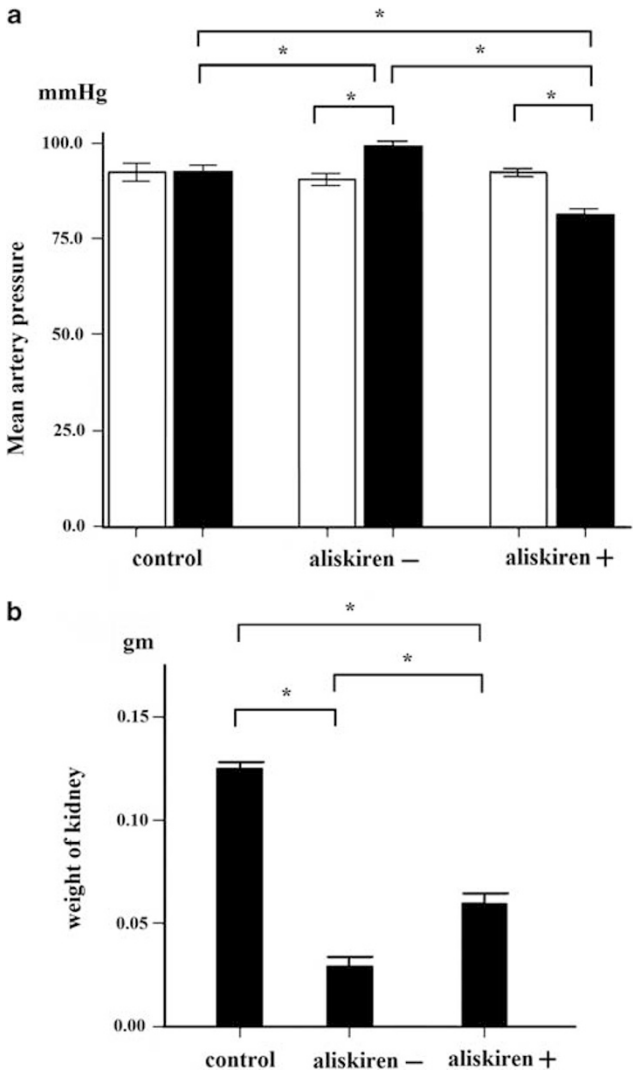

Figure 1 Comparisons of blood pressure and kidney weight. Aliskiren significantly decreased mean blood pressure in mice with RAC (92.1 ± 1.1 *vs.* 81.0 ± 1.8 mm Hg, $P<0.05$) and reduces the atrophic effect of chronic renal ischemia (aliskiren−: 0.03 ± 0.00; aliskiren+: 0.06 ± 0.01g; $P<0.05$). (**a**) The mean arterial pressure (□: before RAC; ■: 3 weeks after RAC). (**b**) Weight of kidneys 3 weeks after RAC (*$P<0.05$).

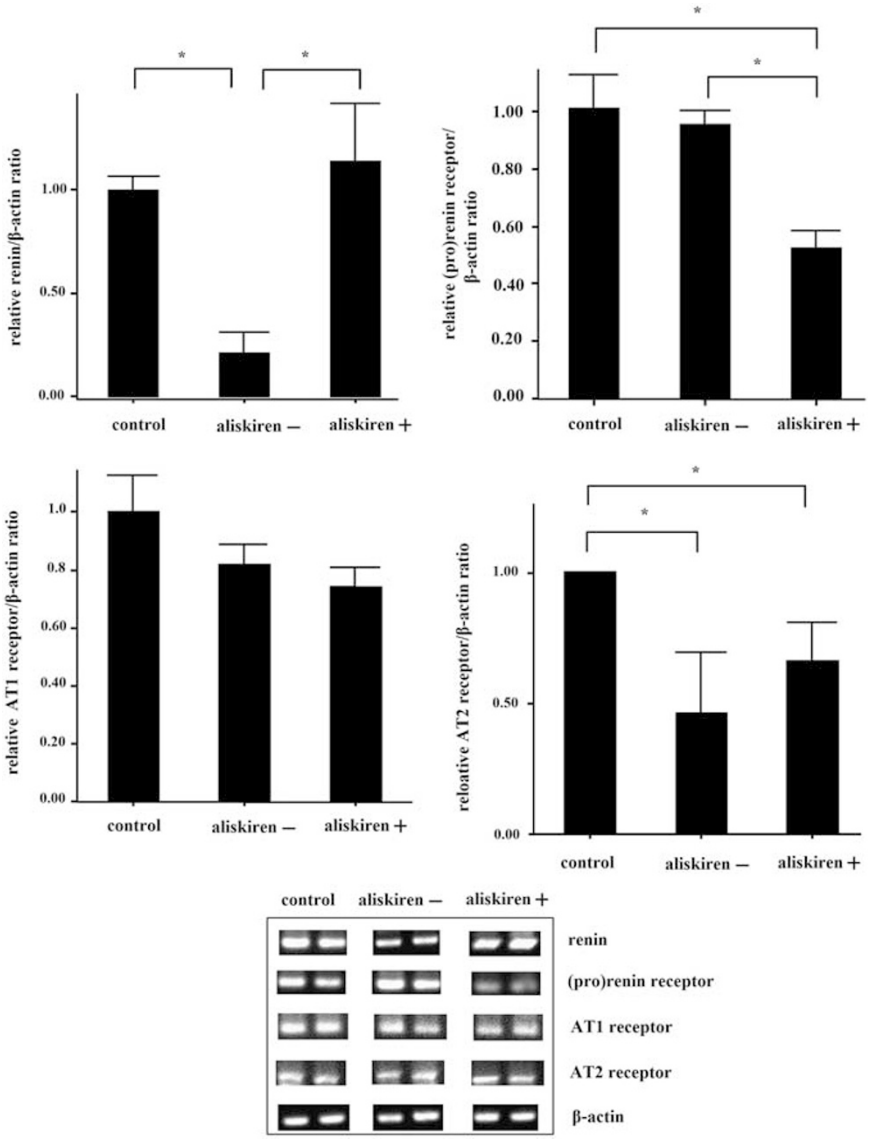

Figure 2 RAAS profiles of ischemic kidneys analyzed by quantitative PCR. Aliskiren treatment significantly increased renin and decreased the (P)RR expression in ischemic kidneys (*$P<0.05$).

Kidneys which underwent RAC were significantly smaller than sham-operated mice; aliskiren reduced the atrophic effect of chronic renal ischemia (control: 0.14 ± 0.00; aliskiren−: 0.03 ± 0.00; aliskiren+: 0.06 ± 0.01 gm, $P<0.05$; Figure 1b).

Effects of aliskiren on kidney RAAS profiles

Chronic renal ischemia decreased renal renin mRNA expression, but did not alter (pro)renin receptor ((P)RR)) and angiotensin II type 1 receptor mRNA expression. AT2 receptor however was significantly decreased in chronic ischemic kidneys. Aliskiren treatment significantly increased renal renin levels and decreased (P)RR mRNA expression in ischemic kidneys. The angiotensin II type 1 and type 2 receptor expression was not significantly altered between kidneys with and without aliskiren treatment (Figure 2).

Aliskiren increased klotho expression

Chronic renal ischemia decreased *klotho* expression significantly (control *vs.* kidney with RAC; real-time PCR: 1.00 ± 0.03 *vs.* 0.05 ± 0.00, $P<0.05$; western blot: 1.00 ± 0.03 *vs.* 0.29 ± 0.07, $P<0.05$; Figure 3). Aliskiren treatment increased *klotho* mRNA and protein expression in ischemic kidneys significantly (aliskiren+ *vs.* aliskiren−; real-time PCR: 0.63 ± 0.08 *vs.* 0.05 ± 0.00, $P<0.05$; western blot: 0.84 ± 0.04 *vs.* 0.29 ± 0.07, $P<0.05$; Figure 3).

Aliskiren decreased Bax and caspase-3 expression, and reduced cell apoptosis

Kidneys with RAC significantly increased levels of Bax and caspase-3 protein expression compared with normal kidneys (Bax: 2.71 ± 0.07 *vs.* 1.00 ± 0.01, $P<0.05$; caspase-3: 5.77 ± 1.01 *vs.* 1.00 ± 0.13, $P<0.05$; Figure 4a). Aliskiren treatment significantly decreased Bax and caspase-3 protein expression in ischemic kidneys compared with the non-treatment group (Bax: 1.73 ± 0.09 *vs.* 2.71 ± 0.07, $P<0.05$; caspase-3: 2.36 ± 0.10 *vs.* 5.77 ± 1.01, $P<0.05$; Figure 4a). TUNNEL staining results showed that the number of TUNNEL-positive cells in renal parenchyma was significantly decreased in RAC mice treated with aliskiren (Figure 4b).

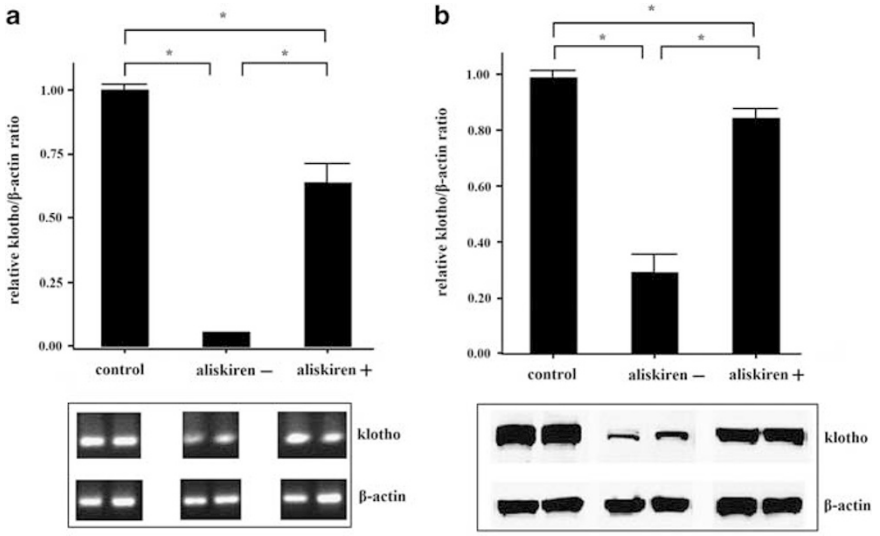

Figure 3 Quantitative PCR and western blot results for *klotho* expression in ischemic kidneys. Aliskiren significantly increased klotho mRNA and protein expression in ischemic kidneys. (**a**) Quantitative PCR results, (**b**) western blot results (*$P<0.05$).

Aliskiren decreased transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF) expression

Chronic renal ischemia increased TGF-β1 and CTGF protein expression when compared with the control mice (TGF-β1: 3.96 ± 0.87 *vs.* 1.00 ± 0.05, $P<0.05$; CTGF: 20.22 ± 6.63 *vs.* 1.00 ± 0.01, $P<0.05$). Aliskiren treatment decreased TGF-β1 and CTGF protein expression in ischemic kidneys when compared with the non-treated mice (TGF-β1: 1.04 ± 0.28 *vs.* 3.96 ± 0.87, $P<0.05$; CTGF: 8.46 ± 2.30 *vs.* 20.22 ± 6.63, $P<0.05$; Figure 5).

Aliskiren decreased collagen I expression, but did not alter collagen III expression

Comparing with the control group, kidneys with RAC showed significantly increased collagen I and III mRNA expression levels (collagen I: 6.03 ± 1.07 *vs.* 1.00 ± 0.03, $P<0.05$; collagen III: 3.77 ± 0.53 *vs.* 1.00 ± 0.11). Aliskiren treatment decreased the collagen I mRNA expression significantly compared with the non-treatment group (aliskiren+ *vs.* aliskiren−: 2.64 ± 0.28 *vs.* 6.03 ± 1.07; $P<0.05$). However, aliskiren treatment did not change the mRNA expression of collagen III and VI in ischemic kidneys (Figure 6).

Aliskiren decreased kidney fibrosis in ischemic kidney

Histochemical staining revealed that chronic renal ischemia caused by RAC led to severe nephrosclerosis and inflammation. Furthermore, even with aliskiren treatment, kidneys with RAC showed prominent kidney fibrosis when compared with sham-operated mice (Figure 7a). Immunostaining revealed that interstitial collagen I deposition was minimal in normal control kidneys (score: 0). In contrast, the intensity of interstitial collagen I deposition in ischemic kidney without aliskiren treatment was stronger (score: 5+). However, treatment with aliskiren reduced the intensity of interstitial collagen I deposition in ischemic kidneys (score: 2+; Figure 7a). The nephrosclerosis scores of study mice were plotted in Figure 7b. The severity of nephrosclerosis was significantly decreased in kidneys with aliskiren treatment compared with those without treatment.

DISCUSSION

Chronic renal ischemia is different from acute ischemia injury.[25,26] Although chronic renal ischemia is more relevant to clinical CKD, it is not as widely studied as acute ischemia injury. Our study revealed that aliskiren treatment attenuates the effect of chronic ischemia on *klotho* expression, apoptosis and renal fibrosis. Aliskiren treatment reduced blood pressure and attenuated renal renin decrement. Our study also revealed that intrarenal renin expression was decreased in chronic ischemic kidneys, which is in agreement to a previous study in rats.[27] The decreased renin expression in chronic ischemic kidneys may come from local chronic ischemia that may destroy the renin-producing cells.[28]

In vivo, (P)RR transgenic rats develop glomerulosclerosis and hypertension in the absence of changes to renin or angiotensin levels.[29] Various animal models demonstrated that inhibition of pro-renin binding to the (P)RR can prevent or even abolish the development of cardiac fibrosis and diabetic nephropathy.[30] In our study, aliskiren significantly decreased renal (P)RR mRNA expression in chronic ischemia. The observed effect may suggest that decreasing renal (P)RR expression has an important role in the protective renal effect of aliskiren.

The *klotho* is an anti-aging gene that is predominantly expressed in the kidney.[31] Renal *klotho* expression was found to decrease in spontaneously hypertensive rats, 5/6 nephrectomized rats, non-insulin-dependent diabetes mellitus rats and in ischemia–reperfusion injury models.[32] In humans, *klotho* expression also decreases in CKD patients,[33] and also has protective renal effects by protecting cells/tissues from oxidative stress and apoptosis.[29] In our study, we demonstrated that direct renin inhibition significantly increased *klotho* expression *in vitro* and *in vivo*. The increased expression of *klotho* gene might further protect chronic ischemic renal injury in animals undergoing aliskiren treatment.

Our results revealed that direct renin inhibition could effectively alleviate the kidney fibrosis caused by chronic kidney ischemia. Aliskiren is designed to block the enzymatic site of human renin. The difference in the amino acid sequence and stereo structure of human and mouse renin could alter the pharmacodynamics of aliskiren.

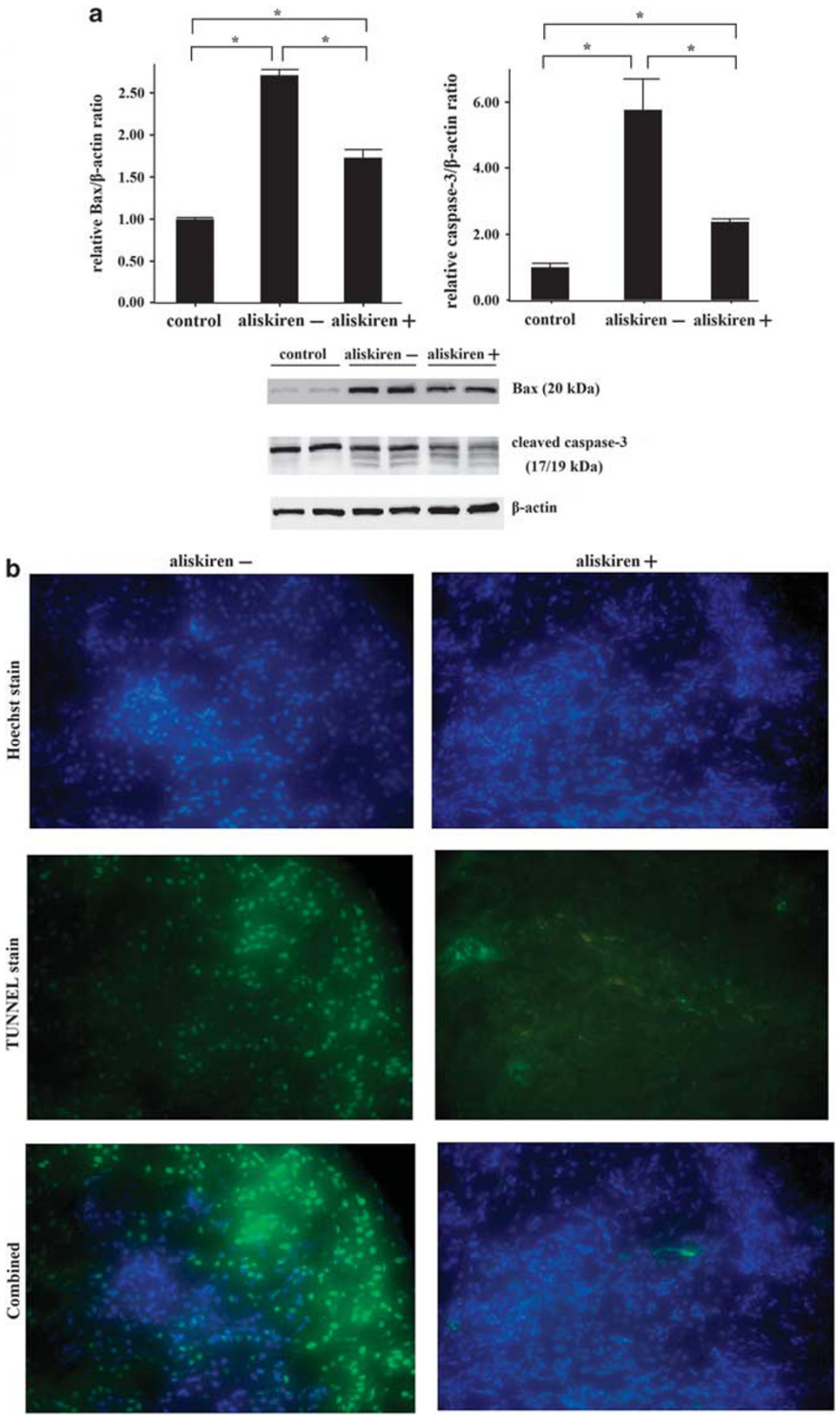

Figure 4 Results of western blotting for Bax and caspase-3, and TUNNEL staining. (**a**) Aliskiren decreases Bax and caspase-3 protein expression in ischemic kidneys significantly. (**b**) The number of TUNNEL-positive cells in renal parenchyma was significantly decreased in RAC mice treated with aliskiren (*$P<0.05$).

Animals expressing human renin gene have often been used as animal models in studies for aliskiren.[34,35] However, recent reports showed that aliskiren still had renal protective effects in the animal models without expressing human renin.[36,37] Our study also showed the similar results. In addition, our data demonstrated that the intrarenal renin expression decreased when chronic renal ischemic injury advanced. Whether mechanisms other than direct renin inhibition had roles in the renal protective effects of aliskiren needs further studies to define.

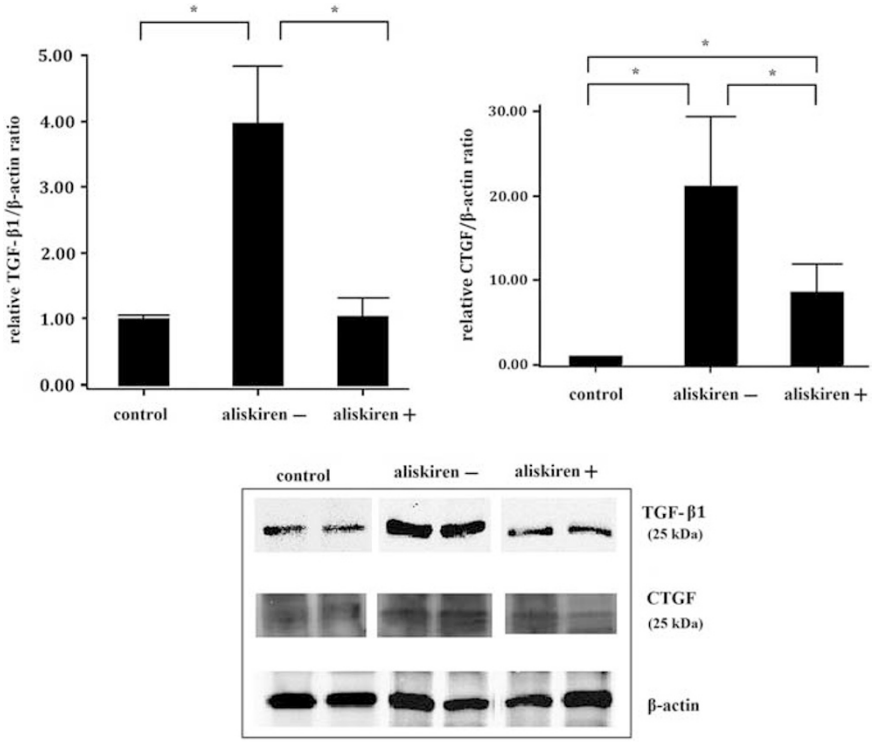

Figure 5 Western blotting for TGF-β1 and CTGF. Aliskiren significantly reduced intrarenal TGF-β1 and CTGF protein expression in ischemic kidneys (*$P<0.05$).

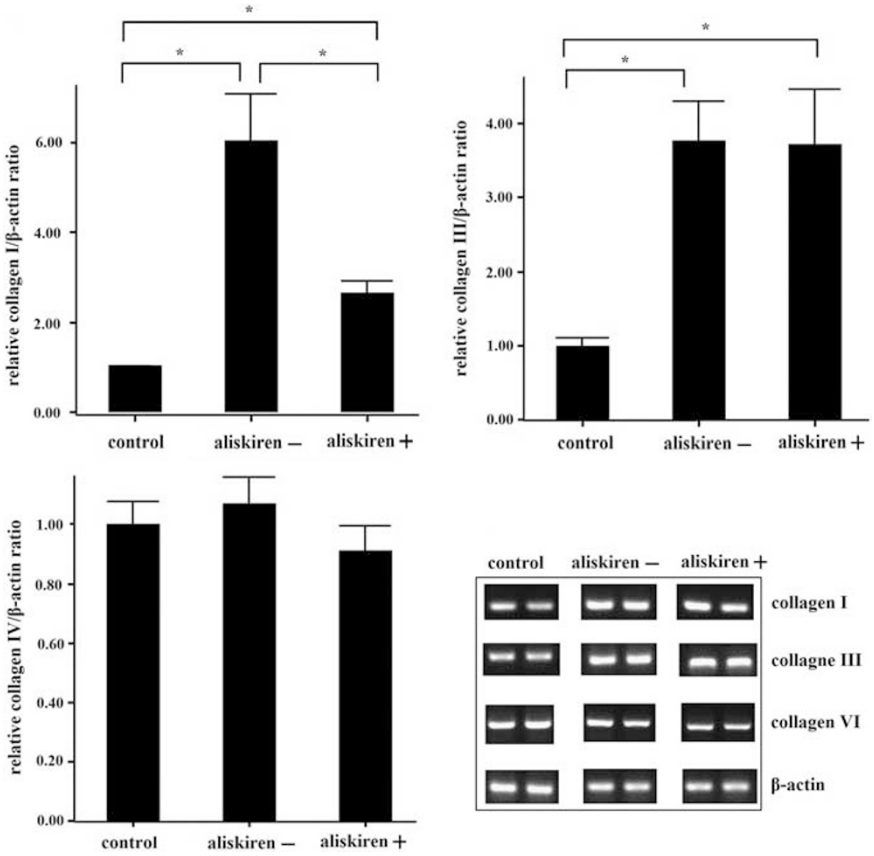

Figure 6 Quantitative PCR for collagen I, III and IV expression. Aliskiren decreased collagen I mRNA expression in ischemic kidney significantly. Collagen III and IV mRNA expression did not significantly differ between treated and non-treated groups (*$P<0.05$).

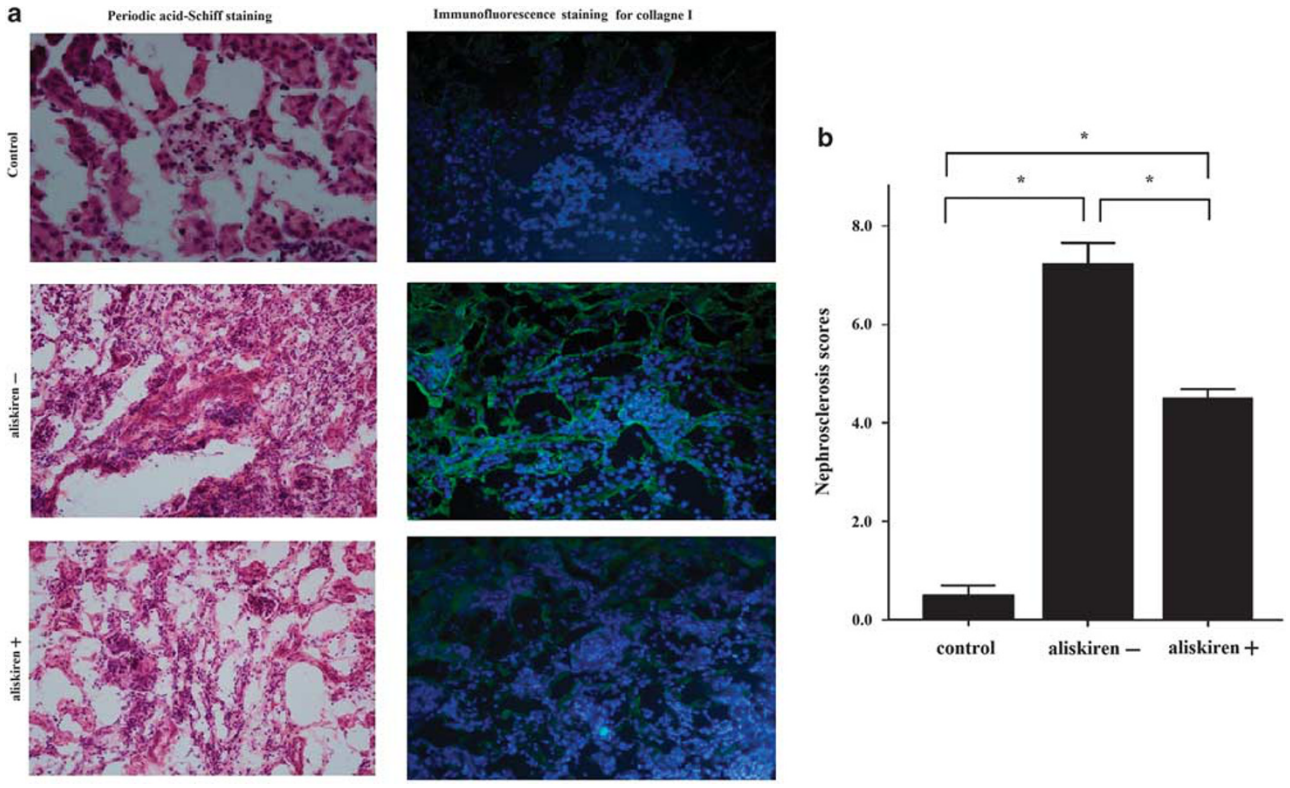

Figure 7 Representative results of pathological staining, and plots of nephrosclerosis scores. (**a**) Results of periodic acid-Schiff and immunofluorescence staining. Aliskiren significantly reduced renal fibrosis in chronic ischemic kidneys. In the normal control mice, only minimal kidney fibrosis and collagen I staining (score: 0) are noted. Experimental mice without aliskiren treatment are associated with marked collagen I staining (score: 5+). Experimental mice with aliskiren treatment are associated with a reduction in the collagen I staining (score: 2+). (**b**) Plots of nephrosclerosis scores. The nephrosclerosis scores of normal control and ischemic kidneys with and without aliskiren treatment were 0.5 ± 0.2, 4.5 ± 0.2 and 7.3 ± 0.4, respectively ($^*P < 0.05$).

There are several limitations in our study. The number of study animals is small, which could limit the reliability of the statistical analysis in this study.

In conclusion, direct renin inhibition with aliskiren could increase *klotho* expression, reduce fibrogenic cytokine production and cell apoptosis, and attenuate the kidney fibrosis in an animal model of chronic ischemic renal injury.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Hypertension Research website (http://www.nature.com/hr)