Original Paper

Rapamycin Attenuates Aldosterone-Induced Tubulointerstitial Inflammation and Fibrosis

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Rapamycin • Aldosterone • Tubulointerstitial Inflammation • Fibrosis

Abstract

Background/Aim: Aldosterone (Aldo), a mediator of kidney fibrosis, is implicated in the pathogenesis of chronic kidney diseases (CKD). The aim of this study was to evaluate the regulatory role of rapamycin (Rap) in Aldo-induced tubulointerstitial inflammation and fibrosis.

Methods: Uninephrectomized, Sprague-Dawley rats were given 1% NaCl (salt) to drink and were randomized to receive treatment for 28 days as follows: vehicle infusion (control), 0.75 µg/h Aldo subcutaneous infusion, or Aldo infusion plus 1 mg/kg/day of Rap by intraperitoneal injection. The effect of Rap on Aldo-induced fibrosis and renal inflammation was investigated using Masson's technique, immunohistochemistry, and western blotting. The effects of Rap on the Aldo-induced epithelial-mesenchymal transition (EMT) process and on TNF-α mRNA expression and secretion in cultured HK-2 cells were investigated by immunofluorescent staining, western blot, qRT-PCR and ELISA. **Results:** An *in vivo* study indicated that signaling by the mammalian target of Rap (mTOR) was activated in rats in the Aldo group compared to controls, as indicated by up-regulated expression of p-mTOR and p-S6K. In addition, the inflammatory response increased, as evidenced by increases in inflammatory markers (MCP-1, ICAM-1, F4/80), and the accumulation of extracellular matrix (ECM), as indicated by increased collagen I and fibronectin expression and pro-fibrogenic gene (PAI-1 and TGF-β1) expression. These changes were attenuated by Rap treatment. An *in vitro* study showed that Rap significantly suppressed the Aldo-induced EMT process and TNF-α mRNA expression and secretion in cultured HK-2 cells. **Conclusions:** Rap can ameliorate tubulointerstitial inflammation and fibrosis by blocking mTOR signaling. Tubular cells may be a major cell type involved in this physiologic process.

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Introduction

Renal inflammation and fibrosis is an important factor in chronic kidney disease (CKD) regardless of the initial cause. The activation of the renin-angiotensin-aldosterone (Aldo) system (RAAS), also an initial cause of renal inflammation and fibrosis, is a major hallmark in the development and progression of organ damage in CKD [1]. Beyond the classical effect of Aldo on sodium reabsorption in the distal nephron, the evidence suggests that Aldo plays an important role in regulating cell growth, inflammation, and fibrosis [2, 3].

The mTOR signaling pathway participates in the activation of macrophages [4, 5] and myofibroblasts [6-8], indicating the importance of mTOR in the regulation of kidney inflammation and fibrosis. Activation of mTOR most prominently results in the phosphorylation of two downstream targets, the ribosomal S6 Kinase (S6K) and 4E-BP (eukaryotic translation-initiation factor 4E-binding protein), which stimulate ribosome biogenesis and translation to increase cell mass [9, 10]. Meanwhile, the epithelial-mesenchymal transition (EMT), a major mechanism of tubulointerstitial fibrosis [11, 12], is tightly associated with mTOR activity. Rapamycin (Rap), an mTOR signaling inhibitor, exerts an anti-fibrosis effect in many tissue fibrosis diseases [13-15]. However, whether Rap exerts a renoprotective effect in Aldo-induced renal injury has not been explored.

In light of these reports, we examined the regulatory role of mTOR signaling in the potential protective effect of Rap on interstitial inflammation and fibrosis in an Aldo-induced rat model. We also investigated the effect of Rap on the expression and secretion of a key inflammatory factor (tumor necrosis factor- α , TNF- α) as well as the EMT process induced by Aldo in cultured proximal tubular cells (HK-2). In this study, we showed that Aldo stimulated renal inflammation and fibrosis *in vivo*, and promoted TNF- α expression and secretion as well as EMT in cultured HK-2 cells. Treatment with Rap improved all these changes.

Materials and Methods

Materials

Reagents were obtained from the following sources. The Aldo, Rap, β -actin and α -smooth muscle actin (α -SMA) antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture materials were purchased from Gibco BRL (Gaithersburg, MD, USA). Collagen I antibody was purchased from Abcam, Ltd. (Cambridge, England). The mTOR, p-mTOR, S6K, and p-S6K antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The transforming growth factor- β 1 (TGF- β 1), plasminogen activator inhibitor (PAI)-1 (PAI-1), fibronectin, monocyte chemoattractant protein (MCP)-1 (MCP-1), F4/80, and intercellular cell adhesion molecule (ICAM)-1 (ICAM-1) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The E-cadherin antibody was purchased from BD Transduction Laboratories (Lexington, KY, USA).

Animal preparation

All experimental procedures were performed according to our previous studies [16]. After 2 weeks of recovery from surgery, an osmotic minipump (model 2004; Alzet, Cupertino, CA) was implanted subcutaneously to infuse vehicle or Aldo (at 0 weeks). The rats were assigned randomly to treated or untreated groups for 4 weeks: group 1, 1% NaCl in the drinking solution plus vehicle (n=8); group 2, 1% NaCl in the drinking solution plus Aldo (0.75 μ g/h, subcutaneously, n=8); group 3, 1% NaCl in the drinking solution plus Aldo plus Rap (1 mg/kg/day, by intraperitoneal injection, n=8).

Histological examination and Immunohistochemistry

The kidneys were fixed with 10% formalin, sectioned into 3 μ m slices and stained with Masson's trichrome stain to assess collagen levels. Immunohistochemical stains were performed on formalin-fixed, paraffin-embedded 3 μ m sections, which were rehydrated, and the antigens were retrieved using heated citrate. The primary antibodies against the following proteins were used: MCP-1 (1:200), F4/80 (1:100), ICAM-1 (1:200), fibronectin (1:200), collagen-I (1:250), TGF- β 1 (1:100), and PAI-1 (1:200). Staining was

visualized using horseradish-peroxidase coupled secondary antibodies (Vectastain elite, Vector Labs). Related isotype immunoglobulins (Jackson ImmunoResearch, USA) were used as the negative controls. All immunohistochemical analyses were repeated at least three times and representative images were presented. All analyses were performed in a masked manner.

The quantitation of immunostaining was carried out on coded slides. The expression of collagen I, fibronectin, MCP-1, ICAM-1, PAI-1, and TGF- β 1 in the entire cortex (a cross-section of the kidney) was determined using an image analyzer version 6.1 (WinRoof) and the data were expressed as a percentage of the positive area as previously described [17-19]. The number of F4/80+ cells in the tubulointerstitium was counted in 20 consecutive fields under $\times 400$ magnification by means of a 0.0625-mm² graticule fitted in the eyepiece of the microscope and expressed as cells per mm².

Cell culture

The HK2 cell line from ATCC (American Type Culture Collection) was cultured in DMEM supplemented with 10% FBS until the cells were 80% confluent. Then, the cells were cultured in DMEM containing different concentrations of Aldo (10^{-9} M- 10^{-7} M), with or without the addition of 1 mM Rap, for the indicated time.

Cytokine detection

TNF- α was measured in the supernatant using ELISA kits (R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured HK-2 cells in respective groups, reverse transcribed to cDNA, and subjected to PCR amplification according to the procedures described previously [20]. The primers were as follows: TNF- α : sense 5'-TTCTCATTCTGCTTGTG-3' and anti-sense 5'-TTGGTGGTTTGCTACG-3'; and GAPDH: sense 5'-AGGTCGGTGTGAACGGATTTG3' and anti-sense 5'-GGGGTCGTTGATGGCAACA3'.

Immunofluorescence Staining

HK-2 cells were fixed on coverslips using cold methanol/acetone (1:1) for 10 min at -20°C, followed by washing three times in PBS. After blocking with 20% normal donkey serum in PBS buffer, the slides were incubated overnight at 4°C with the primary antibodies against human E-cadherin (1: 25) and fibronectin (1:100). For visualization of the primary antibodies, the slides were stained with FITC-conjugated secondary antibodies. Slides were blinded and three random fields were digitized using a Nikon microscope attached to a digital camera. Each experiment was repeated 3 times.

Western Blotting Analysis

Total proteins were extracted using RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, Inc., St. Louis, MO). Protein concentrations were determined using the BCA method (Beyotime Institute of Biotechnology, Haimen, China). Equal amounts of protein were loaded onto each lane, separated by SDS – polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. After blocking with 5% skimmed milk in Tris-buffered saline (TBS) (pH 7.6) at room temperature, the membranes were incubated overnight at 4°C with primary antibodies for mTOR (1:1000), p-mTOR (1:1000), S6K (1:1000), p-S6K (1:1000), E-cadherin (1: 2000), α -SMA (1:1000) and fibronectin (1:100). After incubation with the respective secondary antibody, the immune complexes were detected using ECL western blotting reagents.

Statistical Analysis

The statistical significance of a difference between the control and treatment groups was determined by simple ANOVA followed by Dunnett's multiple comparison tests. Statistical significance was defined as $P < 0.05$.

Results

Rap inhibited the activation of mTOR signaling in rats induced by Aldo

We first examined whether Aldo activated mTOR signaling in kidneys from Aldo-induced rats, and if so, whether Rap, a mTOR signaling inhibitor, could inhibit the activation

Fig. 1. Rap inhibited the mTOR signaling pathway in Aldo-induced rats. (A) Lysates of kidney tissues from various groups were analyzed by western blotting for the detection of mTOR and S6K and their phosphorylation activity. (B) The quantification of the intensity of the phosphorylation of mTOR and S6K. The data are presented after normalization to mTOR or S6K expression, respectively and are expressed as the mean $\pm$ SEM; n=3 per group; #P <0.01 versus controls; *P <0.05 versus Aldo alone.

Fig. 2. Rap attenuated renal interstitial fibrosis in Aldo-induced rats. (A) Kidney sections from various groups were subjected to MTS. Original magnification $\times$ 200. (B) Quantitative determination of renal fibrotic lesions in different groups. Renal fibrotic lesions (defined as percentage of MTS-positive fibrotic area) were quantified on MTS sections by computer-aided morphometric analyses. #P <0.01 versus sham controls; *P <0.05 versus Aldo alone (n = 8).

of the mTOR signaling. Compared with the control group, Aldo activated mTOR signaling. However, Rap inhibited the activation of mTOR signaling compared with Aldo-induced rats (Fig. 1A and B).

Rap attenuated renal interstitial fibrosis in rats induced by Aldo

Because mTOR signaling is highly activated and is involved in Aldo-induced kidney injury, we examined whether Rap could improve Aldo-induced injury. Fig. 2A shows the representative micrographs of kidney sections after Masson's trichrome staining (MTS). Quantitative determination by computer-aided morphometric analyses revealed that Rap reduced renal fibrotic lesions in Aldo-induced rats (Fig. 2B).

Rap inhibited the protein expression of major interstitial matrix genes in Aldo-induced rats

We examined the protein expression of major interstitial matrix genes by immunohistochemistry. As shown in Fig. 3, Rap treatment inhibited the renal expression of collagen I and fibronectin induced by Aldo infusion.

Fig. 3. Rap attenuated the expression of collagen I and fibronectin in Aldo-induced rats. Immunohistochemical staining for collagen I and fibronectin in kidney sections from different groups (A). These areas were also assessed quantitatively (B), as described in *Materials and Methods*. The data are reported as the means $\pm$ SE. #P < 0.01 versus controls; *P < 0.05 versus Aldo alone. Original magnification $\times 400$.

Fig. 4. Rap attenuated the expression of TGF- β 1 and PAI-1 in Aldo-induced rats. Immunohistochemical staining for TGF- β 1 and PAI-1 in kidney sections from various groups (A). These areas were also assessed quantitatively (B), as described in *Materials and Methods*. The data are reported as the means $\pm$ SE. #P < 0.01 versus controls; *P < 0.05 versus Aldo alone. Original magnification $\times 400$.

Rap inhibited the protein expression of fibrogenic genes in Aldo-induced rats

We examined several fibrogenic genes by immunohistochemical staining. Compared with Aldo-induced rats, Rap inhibited tubulointerstitial expression of TGF- β 1 and PAI-1 (two well-known mediators in the pathogenesis of renal fibrosis) (Fig. 4). These results suggested a potential role of Rap in blocking renal fibrosis in an Aldo-induced renal fibrosis model.

Fig. 5. Rap attenuated the expression of MCP-1, ICAM-1, and F4/80+ in Aldo-induced rats. Immunohistochemical staining for MCP-1, ICAM-1, and F4/80+ in kidney sections from different groups (A). These areas were also assessed quantitatively (B), as described in *Materials and Methods*. The data are reported as the means $\pm$ SE. #P < 0.01 versus controls; *P < 0.05 versus Aldo alone. Original magnification $\times 400$.

Fig. 6. Rap inhibited the mTOR signaling pathway in Aldo-induced HK-2 cells. (A) HK-2 cells stimulated with 10^{-7} M Aldo for different time points (6, 12, 24, 48 h) or (B) with different concentrations (10^{-9} , 10^{-8} , 10^{-7} M) of Aldo for 48 h. Western blots were analyzed for S6K and their phosphorylation activity. (C) HK-2 cells were pre-incubated with Rap (1 nM) for 30 min before Aldo (10^{-7} M) treatment for 48 h. The data are representative of three similar experiments and are quantified as the mean $\pm$ SEM. #P < 0.01 versus controls; *P < 0.05 versus Aldo alone.

Rap improved interstitial inflammation in Aldo-induced rats.

Because the progression of renal fibrosis can initially be characterized as an induction of the inflammatory response, ultimately resulting in widespread fibrotic changes, we examined several inflammatory markers by immunohistochemical staining, including

Fig. 7. Rap inhibited TNF- α expression and secretion in Aldo-induced HK-2 cells. (A) Effects of Aldo on tumor necrosis factor (TNF)- α mRNA levels in human proximal tubular cells (B) and protein levels in the culture medium in the absence or presence of Rap. The data are expressed as the mean $\pm$ SEM; $n=6$ per group; [#] $P < 0.01$ versus controls; ^{*} $P < 0.05$ versus Aldo alone.

F4/80 (macrophages marker), intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1). Our results revealed that the administration of Rap significantly reduced the interstitial infiltrates of F4/80 macrophages, ICAM-1, and MCP-1 (Fig. 5) compared with Aldo-induced rats.

Rap inhibited Aldo-induced activation of the mTOR signaling pathway in cultured HK-2 cells

To determine whether the tubular epithelial cells remain potential targets of Rap in Aldo-induced renal fibrosis, we evaluated the mTOR signaling changes in cultured proximal tubular cells. As shown in Fig. 6A, Aldo increased the level of p-S6K at 12 h, with maximal stimulation at 48 h. Therefore, the 48 h time point was used in subsequent experiments to determine the concentration response of mTOR signaling to Aldo stimulation. As shown in Fig. 6B, Aldo increased p-S6K in a concentration-dependent manner, with maximal stimulation at 10^{-7} M. As expected, the increased expression of p-S6K can be significantly attenuated by the co-administration of Rap (Fig. 6C).

Rap attenuated Aldo-induced TNF- α expression and secretion in cultured HK-2 cells

Because Rap attenuated Aldo-induced renal inflammation *in vivo*, we investigated whether the tubular epithelial cells are involved in this process *in vitro*. In our study, Aldo treatment for 48 h significantly activated increased TNF- α mRNA, and also increased TNF- α level in the culture medium. These changes in mRNA and secreted protein levels were suppressed by co-treatment with Rap (Fig. 7A and B).

Rap inhibited Aldo-induced EMT in HK-2 cells

Because EMT plays an important role in the genesis and development of interstitial fibrosis, we studied the relationship between EMT and mTOR signaling in HK2 cells. As shown in Fig. 8A, control HK-2 cells exhibited the typical cobblestone morphology of epithelial cells when grown in culture, while the cells showed profound morphologic changes after Aldo treatment for 48 h, with cells displaying an elongated and fibroblast-like morphology. Simultaneous incubation of Rap with Aldo inhibited the change to the myofibroblast phenotype and largely restored the epithelial morphology of the HK-2 cells.

Additionally, Rap inhibited Aldo-driven suppression of E-cadherin and the induction of fibronectin, as shown by immunofluorescent staining (Fig. 8B). Western blot analyses assessing E-cadherin, fibronectin, and α -SMA expression after various treatments produced similar results (Fig. 8C).

Fig. 8. Effect of Rap treatment on EMT of HK-2 cells. (A) Morphological changes. The cells were grown in 6-well plates until 80% confluence and then treated with vehicle and Aldo (10^{-7} M) with or without the addition of 1 mM Rap for 48 h. Photographs were taken using a Nikon microscope (phase contrast). (B) Cell lysates were immunoblotted with antibodies against E-cadherin, α -SMA, fibronectin, vimentin, and β -actin. Graphic presentation of E-cadherin, α -SMA, and fibronectin protein abundances as indicated. Relative E-cadherin, α -SMA, and fibronectin levels were reported after normalizing to β -actin. The data are presented as the means $\pm$ SEM of three experiments. #P < 0.01 versus controls; *P < 0.05 versus Aldo alone. (C) Representative fluorescence staining of E-cadherin and fibronectin. Original magnification $\times 400$.

Discussion

Renal fibrosis is a common final outcome of many different progressive CKDs. The activation of RAAS is an important factor in the development and progression of organ damage in CKDs. In humans, RAS blockade with angiotensin-converting enzyme inhibitor (ACEi) or AngII receptor antagonists (ARB) results in an incomplete suppression of serum Aldo levels, a phenomenon known as “Aldo escape”, which is associated with enhanced excretion of urinary albumin and a decline in GFR [20].

In vitro studies have suggested that Aldo could directly promote fibrotic responses, enhance the production of transforming growth factor TGF- β 1, PAI-1 and connective tissue growth factor [21, 22], and induce EMT in cultured kidney tubular cells, resulting in their transformation into myofibroblasts [23]. These findings suggest that Aldo may lead to matrix accumulation in kidney. Other evidence suggests that inhibition of the mineralocorticoid receptors (MRs) by spironolactone might also provide a therapeutic strategy as an antifibrotic agent. However, the effect of spironolactone in many patients is still unsatisfactory, and spironolactone also results in adverse effects, including hyperkalemia, hyponatremia, gynecomastia, impotence, menstrual disturbances, hirsutism, and decreased libido [24]. Thus, it will be important to develop more effective approaches for suppressing hyperactive RAAS-induced renal fibrosis.

Because aberrant activation of mTOR signaling occurs in the fibrotic kidneys [25-27], the pathway could be exploited as a potential target for anti-fibrosis therapy. In the study presented here, we confirmed that Rap, a specific mTOR signaling inhibitor, could inhibit

Aldo-induced activation of mTOR signaling both *in vitro* and *in vivo*. As expected, Rap improved renal fibrotic lesions in an Aldo-induced rat model, as indicated by the reduced accumulation of ECM and pro-fibronectin genes. These findings are in line with a recent report that blockade of mTOR signaling prevents renal fibrosis in UUO rats [14].

Because infiltration of inflammatory cells is an early and characteristic feature of renal fibrosis [14], and mTOR signaling may play an important role in the initiation and progression of kidney inflammation, we tested whether Rap exerted a protective effect in Aldo-induced renal inflammation. As expected, Rap ameliorated interstitial inflammation as evidenced by reductions in inflammatory markers (MCP-1, ICAM-1, F4/80) compared with Aldo-infused rats. Additionally, some evidence suggests that tubular epithelial cells play an active role in the progression of renal fibrosis via the generation of various pro-inflammatory and pro-fibrotic factors, including cytokines, growth factors and matrix proteins [28-30]. To determine whether Rap protected the kidney from fibrosis partly by inhibiting the fibrogenic role of tubular epithelial cells, we investigated the expression and secretion of TNF- α in Aldo-induced HK-2 cells in the presence or absence of Rap. Our results indicated that Rap inhibited Aldo-induced TNF- α synthesis and secretion in proximal tubular cells. We also confirmed that Rap markedly inhibited the Aldo-induced EMT process, which indicated that inhibiting the EMT process might partially participate in the anti-fibrosis effect of Rap.

Taken together, our studies revealed that Rap could ameliorate interstitial inflammation and fibrosis by inhibiting mTOR signaling. Proximal tubular cells may be one of the major cells in this physiologic process.

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