Amiloride off-target effect inhibits podocyte urokinase receptor expression and reduces proteinuria

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Abstract

The urokinase receptor (uPAR) and its soluble form play a key role in the pathogenesis of focal segmental glomerulosclerosis (FSGS). The modification of uPAR pathological actions on podocytes will become an important task for the development of improved nephroprotective therapeutics. Here we show that podocyte uPAR expression can be reduced using amiloride. Amiloride has a significant role in the reduction of podocyte cell motility in vitro and proteinuria in mice. Amiloride inhibited the induction of uPAR protein and PLAUR messenger RNA (encoding uPAR) and with that it reduced uPAR-mediated β3 integrin activation in lipopolysaccharide (LPS)-treated podocytes. Transwell migration assay and wound healing assay showed that directed and random podocyte motility of LPS-treated podocytes were increased and substantially reduced by amiloride. The off-target effect of amiloride was independent of its function as epithelial sodium channel blocker and different from triamterene. Amiloride was also effective in the LPS mouse model of transient proteinuria (LPS mice) and in the 5/6 nephrectomy rat FSGS model (NTX) by significantly inhibiting podocyte uPAR induction, reducing proteinuria. In addition, amiloride attenuated glomerulosclerosis, as determined by glomerulosclerotic index. Thus, our observations show that amiloride inhibits podocyte uPAR induction and reduces proteinuria in NTX rats and LPS mice. Given the pathological relevance of the uPAR-β3 integrin signaling axis in FSGS, amiloride may be utilized in patients with FSGS.

Keywords: amiloride; β3 integrin; podocyte; proteinuria; urokinase receptor

Introduction

Amiloride, a potassium-sparing diuretic, works by directly blocking the epithelial sodium channel (ENaC) in the collecting ducts in the kidneys [1]. Additionally, amiloride inhibits the Na+/H+ and Na+/Ca2+ antiporters [2], the urokinase receptor (uPAR) expression in tumor-infiltrating lymphocytes [3] and colon cancer cells [4, 5]. uPAR is a glycosylphosphatidylinositol-anchored protein that has been shown to be a proteinase receptor for urokinase but has also been involved in non-proteolytic pathways, mainly through its ability to form signaling complexes with other transmembrane proteins such as integrins, caveolin and G-protein-coupled receptors [6]. uPAR has important roles in wound healing, inflammation and stem cell mobilization as well as in severe pathological conditions such as HIV-1 infection, tumor invasion and metastasis [7]. It also plays a major role in focal segmental glomerulosclerosis (FSGS) through both its membrane-bound form [8] and as circulating FSGS factor [9]. The ability of amiloride to inhibit uPAR induction in lymphocytes and cancer cells is of particular interest given recent evidence of the involvement of uPAR-β3 integrin signaling and in the development of kidney podocyte dysfunction and proteinuria [8, 9].

It has been shown that uPAR interacts with several integrins that modulate their ligand-binding activities [10]. Lipid raft-associated uPAR and β3 integrin place themselves in podocytes and thus form a complex with β3 integrin, thereby causing its activation. It is a key signal that mediates uPAR-induced cellular events leading to proteinuria; the expression of an active β3 integrin is sufficient to induce proteinuria, even in the absence of uPAR. Proteinuria caused by uPAR-β3 integrin signaling can be prevented and reduced by cycloRGDfV [8], a selective inhibitor of β3 integrin [11].

Podocytes, endothelial cells and the glomerular basement membrane (GBM) constitute the kidney filtration barrier, a highly specialized structure for selective ultrafiltration. The common denominator in a variety of kidney diseases is podocyte dysfunction involving proteinuria [12, 13]. Most cases of proteinuria are associated with effacement (retraction) of podocyte foot processes, which represents the motility of podocytes [14]. There, podocytes stay attached to the GBM, but changes in altered podocyte foot process dynamics result in foot process effacement and proteinuria.
In some forms of inflammatory glomerular diseases, such as crescentic glomerulonephritis, podocytes can move out of their microenvironment into areas of crescentic glomerular damage [15, 16]. Interestingly, it has also been reported that amiloride completely prevented pulmonary metastases when given to rats in their drinking water [4, 5]. This amiloride-induced antimetastatic action was reported to be linked to its inhibitory effects on uPAR expression [7, 17, 18].

A previous study reported that amiloride retarded the onset of proteinuria in saline-drinking stroke-prone spontaneously hypertensive rats (SHRSP) [19]. However, this antiproteinuric effect amiloride afforded in the SHRSP cannot be readily explained by its classic therapeutic actions, such as inhibition of the ENaC of the kidney collecting ducts and other actions of amiloride cannot be excluded. In the present study, we show that amiloride affects uPAR expression in cultured differentiated podocytes, but not triamterene, an ENaC blocker [20]. Furthermore, we show that amiloride affects uPAR-mediated disease in LPS-treated mice as well as in 5/6 nephrectomized rats. Our results provide the experimental evidence in support of an antiproteinuric effect of amiloride, which may be partially attributable to the drug’s off-target effect on inhibition of uPAR in kidney podocytes.

**Materials and methods**

**Proteinuric animal models and glomerulosclerotic analysis**

All animal experiments were performed with the approval of the GGH Animal Care and Use Committee in accordance with the NIH guidelines for the conduct of experiments on animals. The 5/6 nephrectomy rat model (NTX rats) was induced in male Sprague–Dawley rats (initial weight, 250–300 g) by performing surgical resection. One week later, all animals having undergone 5/6 renal mass reduction were then randomized to (i) receive amiloride (A7410-1G; Sigma–Aldrich, USA) once daily by gastric gavage (3 mg kg⁻¹ day⁻¹); (ii) receive triamterene (T4143-10G; Sigma–Aldrich) once daily by gastric gavage (25 mg kg⁻¹ day⁻¹); (iii) or receive vehicle (equal volume of sterile amiloride-free saline, NTX group, n = 14). The rats of sham-operated group (Sham group, n = 14) also received vehicle once daily by gastric gavage. We collected urine at time points of 2, 4, 8 and 12 weeks for Bradford protein analysis (Bradford protein assay kit, Life Sciences, USA).

The extent of glomerulosclerosis was determined in 3 mm kidney sections and a glomerulosclerotic index was then calculated, as described previously [21]. In brief, 60 glomeruli from each rat were examined in a masked protocol. The degree of sclerosis in each glomerulus was graded on a scale of 0–4 as described previously with Grade 0, normal; Grade 1, sclerotic area up to 25% (minimal); Grade 2, sclerotic area 25–50% (moderate); Grade 3, sclerotic area 50–75% (moderate to severe) and Grade 4, sclerotic area 75–100% (severe).

For the LPS mouse model of transient proteinuria (LPS mice) [8, 22], we injected C57BL/6 mice intraperitoneally with 200 μg LPS (L-2880; Sigma–Aldrich) in a total volume of 500 μL. Controls (Con group, n = 5) received the same volume of sterile LPS-free saline. After LPS injection, mice were gavaged with saline alone (LPS group, n = 7), amiloride once daily (5 mg kg⁻¹ day⁻¹), LPS + amiloride (5) group, n = 6; 10 mg kg⁻¹ day⁻¹, LPS + amiloride (10) group, n = 6; 50 mg kg⁻¹ day⁻¹, LPS + amiloride (50) group, n = 6) and triamterene once daily (25 mg kg⁻¹ day⁻¹, LPS + triamterene (25) group, n = 6; 50 mg kg⁻¹ day⁻¹, LPS + triamterene (50) group, n = 6). We collected urine for a Bradford assay 24 h after LPS injection.

**Cell culture**

Conditionally immortalized mouse podocytes were kindly provided by Dr P. Mundel (Massachusetts General Hospital, Boston, USA) and cultured as reported previously [23]. To propagate podocytes, cells were cultivated on BD BioCoat Collagen I plates (BD Biosciences, USA) at 33°C in the presence of 20 U/mL mouse recombinant interferon (IFN)-γ (CTY-358; ProSpec-Tany TechnoGene Ltd, Israel) to enhance expression of a thermosensitive T antigen. To induce differentiation, podocytes were maintained at 37°C without IFN-γ for 10–12 days. To quiescent the cells, differentiated podocytes were serum-starved overnight before experiment.

**Flow cytometry**

The flow cytometry assay was used to assess uPAR induction and active beta 3 integrin on the cell surface of podocytes during the treatment with LPS alone (50 μg mL⁻¹), LPS (50 μg mL⁻¹) plus amiloride (5–100 μg mL⁻¹) or LPS (50 μg mL⁻¹) plus triamterene (0.25–25 μg mL⁻¹). After 24 h of these treatment, 10⁶ LPS⁻¹ cells were trypsinized, washed with phosphate-buffered saline (PBS) (Ca²⁺ free) and incubated with anti-mouse uPAR antibody (AF-534; R&D Systems, USA) or murine anti-active beta 3 integrin (AP5 antibody; Genetic Testing Institute, USA) for 20 min at 4°C. Cells were then washed in PBS (Ca²⁺ free) and incubated with rabbit anti-goat-Ig fluorescein isothiocyanate (FITC) (sc-2777, Santa Cruz Biotechnology, USA) or goat anti-mouse Alexa Fluor 488 (A11001; Invitrogen, USA) for 20 min at 4°C. Data were collected by Cell Lab Quanta™ SC Flow Cytometry System and analyzed using Cell Lab Quanta™ SC analyze (Beckman Coulter, Inc, USA).

**Immunocytochemistry**

Murine kidneys and cultured podocytes were harvested and snap frozen according to standard protocols and fixed for sectioning in ice-cold acetone for 10 min. For immunofluorescent labeling, sections were washed once with PBS, permeabilized with 0.5% Triton X-100 in PBS and incubated with blocking solution (5% bovine serum albumin) for 20 min at room temperature, before further incubation with one of the primary antibodies (synaptophysin, anti-vimentin, PTEN, p-ERK, p-AMPK, p-AKT, AP5 antibody, Genetic Testing Institute, USA) for 2 h at room temperature. For double labeling, sections were washed three times with PBS for 5 min, and one of the secondary antibodies (goat anti-mouse Alexa Fluor 635, A31575; goat anti-rabbit Alexa Fluor 546, A11010; Invitrogen; rabbit anti-goat-Ig FITC, sc-2777, Santa Cruz Biotechnology) was applied for 2 h. Pictures were captured with confocal microscopy (Leica Microsystems). All images were analyzed by two investigators blinded to the identity of the samples.

**Real-time quantitative polymerase chain reaction**

Real-time quantitative polymerase chain reaction (PCR) for cultured podocytes and kidney cortex isolated from rodent proteinuric models was performed as recommended in PrimeScript™ RT reagent Kit (Takara Bio Inc, Japan) using the following primers: rat PLAUR forward, AGATGTGCTGTTG-GAAACCG; reverse, CAGGGAGGCAATGAGGAT) yielding a 196 bp product; mouse PLAUR (forward, AAGCCTGCAATGCGCTATC; reverse, GGGTGTAGTGTGACACTTACAGGA) yielding a 182 bp product.

**Transwell migration assay**

Transwell cell culture inserts (pore size 5 μm; Costar Corporation, USA) were coated with vitronectin, rinsed once with Dulbecco’s Phosphate-Buffered Saline and placed in Dulbecco’s Modified Eagle’s Medium (10% fetal bovine serum) in the lower compartment. For each experiment, 1 × 10⁴ LPS⁻¹ cultured differentiated podocytes were seeded in the inserts and allowed to migrate for 24 h while being incubated at 37°C. Non-migratory cells were removed from the upper surface of the membrane, and migrated cells were fixed with 4% paraformaldehyde and stained with Crystal Violet Solution (Sigma–Aldrich). The number of migrated cells was counted using phase contrast microscopy with a ×20 objective on a microscope (Olympus) in the center of a membrane (one field). The data presented represent the mean ± SD of four independent experiments.

**Wound healing assay**

Differentiated podocytes (each 1 × 10⁴ LPS⁻¹) were seeded overnight on vitronectin-coated coverslips in six-well plates. Each coverslip
was then scratched with a sterile 200 μL pipette tip, washed with PBS and placed into fresh medium. After 24 h, the cells were fixed with cold methanol, permeabilized with 0.5% Triton X-100 in PBS and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics). Pictures were captured by phase contrast microscopy under a ×10 objective on a microscope (Leica Microsystems) at 0 and 24 h after scratching, and the number of cells that had migrated into the same-sized square fields was counted. The data presented represent the mean ± SD of five independent experiments.

Statistical analysis

We assessed statistical significance by one-way analysis of variance followed by least significant difference test for comparison between two groups. P <0.05 was considered significant. All values are expressed as mean ± SD.

Results

Amiloride inhibits uPAR induction in podocytes in vitro

To understand whether amiloride could inhibit uPAR induction in podocytes, we treated cultured differentiated podocytes [23] with LPS alone (50 μg mL⁻¹), LPS (50 μg mL⁻¹) plus amiloride (5–100 μg mL⁻¹) or LPS (50 μg mL⁻¹) plus triamterene (0.25–25 μg mL⁻¹), an ENaC blocker [20]. As expected, an increased induction of uPAR protein expression (Figure 1A and B) and PLAUR messenger RNA (mRNA) expression (Figure 1C) was observed in

Fig. 1. Amiloride treatment inhibits uPAR induction in podocytes in vitro. (A) Immunofluorescence staining for uPAR protein in cultured differentiated podocytes. LPS-treated podocytes showed an increased expression of uPAR protein, and when treated with amiloride, podocytes showed a significant reduction in uPAR expression. In contrast, podocytes treated with triamterene did not show any reduction in uPAR expression. (B) Flow cytometry for uPAR antibody binding to the cultured differentiated podocytes. Flow cytometry of uPAR cell surface expression after LPS treatment shows a high uPAR population. Treatment with amiloride (5–100 μg mL⁻¹), but not triamterene (0.25–25 μg mL⁻¹), reduced uPAR cell surface expression in a dose-dependent manner. (C) Quantitative real-time RT–PCR was performed on cultured differentiated podocytes. PLAUR mRNA expression in LPS-treated podocytes was upregulated. Treatment with amiloride (5–100 μg mL⁻¹), but not triamterene (0.25–25 μg mL⁻¹), inhibited PLAUR mRNA expression in a dose-dependent manner. All values are expressed as means ± SD. *P <0.05, **P <0.01 versus LPS-treated podocytes; ###P <0.01 versus control.
the podocytes treated with LPS. In contrast, podocytes treated with amiloride, but not the ENaC blocker triamterene, showed a significantly decreased expression of uPAR protein (Figure 1A and B) and PLAUR mRNA (Figure 1C). These data show that amiloride inhibited the induction of uPAR expression in podocytes in vitro.

**Amiloride inhibits podocyte motility**

uPAR, a molecule associated with cell motility of podocytes [6, 24], is highly expressed on motile cells. Most cases of proteinuria are associated with effacement (retraction) of podocyte foot processes, which represents a surrogate for podocyte motility [14, 25]. To better understand the inhibition of uPAR induction in podocytes afforded by amiloride, we examined the effect of amiloride on cell motility of podocytes in vitro. We first studied podocyte motility before and after amiloride treatment by transwell migration assay to assess the random migration of differentiated podocytes on vitronectin, a known binding partner of uPAR [26]. LPS treatment for 24 h significantly promoted the migration of podocytes (Figure 2A and B). In contrast, treatment with amiloride, but not triamterene, decreased the number of migrating podocytes (Figure 2A and B).

We also analyzed the effect of amiloride on the spatial motility of podocytes with a scrape-wound assay [27]. Compared to control cells, LPS treatment significantly promoted podocyte wound closure (Figure 2C and D). In contrast, treatment with amiloride, but not triamterene, reduced podocyte-directed motility (Figure 2C and D). Together, these data show that amiloride inhibits podocyte motility, a surrogate indicator for proteinuria and effacement of podocyte foot processes in vivo.

**Amiloride ameliorates proteinuria and glomerulosclerosis in animals**

To understand whether amiloride exerts its effect on proteinuria in animals, we first used amiloride in 5/6 nephrectomy rats (NTX rats), a well-characterized model of non-

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**Fig. 2. Amiloride treatment inhibits podocyte motility.** (A and B) Transwell migration assay of podocytes grown on vitronectin. LPS treatment for 24 h promoted podocyte migration. Treatment with amiloride, but not triamterene, reduced podocyte migration (C and D) Wound healing assay of podocytes grown on vitronectin. Twenty-four hours after scraping of the podocyte cell layer, cells have started to migrate into the wound track. Treatment with LPS significantly enhanced directed podocyte motility. Treatment with amiloride, but not triamterene, reduced directed motility. All values are expressed as means ± SD. **P < 0.01 versus LPS-treated podocytes; ##P < 0.01 versus control.
inflammatory proteinuric kidney disease (for example FSGS) in which critical nephron loss leads to progressive renal dysfunction [28]. In this model, proteinuria and podocyte dysfunction have been well documented [29, 30]. We fed male Sprague–Dawley 5/6 nephrectomy rats once daily with vehicle, triamterene (25 mg kg\(^{-1}\) day\(^{-1}\)) or amiloride (3 mg kg\(^{-1}\) day\(^{-1}\)) and left sham-operated rats with vehicle. Vehicle-treated NTX rats developed heavy proteinuria compared with vehicle-treated sham-operated rats (Figure 3A). Meanwhile, amiloride attenuated proteinuria at time points of 8 and 12 weeks (Figure 3A) (amiloride-treated NTX rats at 8 weeks: 109.22 ± 31.26 mg/24 h versus vehicle-treated NTX rats at 8 weeks: 151.89 ± 40.31 mg/24 h, P < 0.01; Figure 3A; amiloride-treated NTX rats at 12 weeks: 121.37 ± 31.14 mg/24 h versus vehicle-treated NTX rats at 12 weeks: 188.31 ± 29.82 mg/24 h, P < 0.01; Figure 3A). In contrast, triamterene, an ENaC blocker, failed to reduce proteinuria, suggesting that, although amiloride’s classic therapeutic action is to block ENaC, which is the same for triamterene, the ability of amiloride to reduce proteinuria in NTX rats may be mediated by another mechanism.

It has been shown that amiloride, a potassium-sparing diuretic, exerts a modest effect on blood pressure [19]. To rule out the possibility that amiloride may exert its effect on proteinuria by lowering systemic blood pressure, we developed this NTX model by surgical kidney mass reduction instead of ligation of the renal arterial branches, given that the former method results in minimal elevations of blood pressure [31]. Our results showed no significant difference in systolic blood pressure among vehicle-treated NTX rats, triamterene-treated NTX rats and amiloride-treated NTX rats (Figure 3C), suggesting that the antiproteinuric action of amiloride in NTX rats is not hemodynamic.

We next wondered whether amiloride exerts its antiproteinuric effect via its action on podocytes, not other cell types in the kidney. We used LPS-mediated proteinuria mouse model (LPS model) [22]. The concept that LPS causes proteinuria by targeting podocytes and not other cell types in the kidney is in keeping with the observation that podocyte-specific expression of cathepsin L-resistant dynamin [32] or synaptopodin [33] is sufficient to safeguard against LPS-induced proteinuria [34]. Thus, the LPS model is a valuable tool to study podocyte-specific proteinuria. We fed LPS-injected C57BL/6 mice with either vehicle, triamterene (25–100 mg kg\(^{-1}\) day\(^{-1}\)) or amiloride (5–50 mg kg\(^{-1}\) day\(^{-1}\)) and left control mice with vehicle. Compared with vehicle-treated control mice, vehicle-treated LPS mice developed proteinuria (Figure 3B). In contrast, proteinuria in amiloride-treated LPS mice was significantly lower than in vehicle-treated LPS mice (P < 0.01; Figure 3B), suggesting that amiloride attenuates proteinuria in LPS mice via its action on podocytes. Meanwhile, triamterene, an ENaC blocker, did not show any antiproteinuric effect (Figure 3B).

Given the effectiveness, albeit incomplete, of amiloride in reducing proteinuria, the renoprotective effects would need to be examined by light microscopy in an animal model. We, therefore, sought to examine the effect of amiloride on glomerulosclerosis in TNX rat model. Compared with sham rats (Figures 4A), sub-total nephrectomy led to significant glomerulosclerosis (Figure 4B), a histopathological characteristic of TNX rats, especially at the time point of 12 weeks. Treatment of TNX rats with amiloride, but not triamterene, reduced glomerulosclerosis (Figure 4C–E), indicating the renoprotective effect of amiloride may be associated with its anti-proteinuric action.

Fig. 3. Amiloride treatment ameliorates proteinuria in 5/6 nephrectomy rats (NTX rats) and LPS-induced proteinuria mice (LPS mice). (A) At time points of 8 and 12 weeks, the urinary protein loss (proteinuria) in amiloride-treated NTX rats were significantly lower than in untreated NTX rats. In contrast, triamterene-treated NTX rats did not show any significant reduction in proteinuria. (B) Compared with untreated LPS mice, amiloride-treated LPS mice showed a significant reduction in proteinuria. In contrast, triamterene-treated LPS mice did not show any significant reduction in proteinuria. (C) Surgical resection-induced NTX rats showed minimal elevations in systolic blood pressure, and there was no statistical difference in systolic blood pressure among untreated NTX rats, amiloride-treated NTX rats and triamterene-treated NTX rats. All values are expressed as means ± SD. *P < 0.05, **P < 0.01 versus untreated NTX rats or untreated LPS mice; ##P < 0.01 versus sham rats or control mice.
Amiloride treatment inhibits uPAR induction in podocytes in vivo

We then asked whether induction of uPAR expression in the NTX rats and the LPS mice. Morphologically, there was low expression of uPAR in glomeruli from the sham rats (Figure 5A) or the control mice (Figure 5B). uPAR was partially localized in podocytes, as indicated by colabeling with the podocyte marker synaptopodin [24]. In contrast, the expression of uPAR protein in the NTX rats (Figure 5A) and the LPS mice (Figure 5B) was substantially increased in podocytes, indicating that both proteinuric models replicate an induction of uPAR expression in podocytes during human proteinuric kidney diseases, as previously reported for FSGS and diabetic nephropathy [8]. Interestingly, after amiloride treatment, we found a substantial reduction of uPAR protein expression in both NTX rats (Figure 5A) and LPS mice (Figure 5B). In contrast, triamterene failed to inhibit uPAR protein expression in both models. We then performed real-time quantitative PCR with kidney cortex isolated from these two proteinuric animal models. We analyzed PLAUR (encoding uPAR) expression in RNA samples from NTX rats or LPS mice. We found low level PLAUR mRNA expression in control rats or mice. In contrast, NTX rats and LPS mice had a significant increase in PLAUR mRNA expression (Figure 5C and D). Notably, we found that, NTX rats and LPS mice, when treated with amiloride, showed a reduced PLAUR mRNA expression (Figure 5C and D). However, triamterene failed to inhibit PLAUR mRNA expression in these two proteinuric animal models.

Amiloride treatment inhibits activation of β3 integrin in podocytes in vivo and in vitro

It is known that uPAR interacts with several integrins that modulate their ligand-binding activities. β3 integrin is among those that are modulated by uPAR, and activation of β3 integrin in glomeruli is decreased in uPAR-deficient mice [8]. Recent evidence showed that the activation of β3 integrin is a key signal that mediates uPAR-induced cellular events leading to proteinuria, and the expression of a constitutively active β3 integrin is sufficient to induce proteinuria [8, 9]. We wondered whether amiloride reduces activation of podocyte β3 integrin, given that the induction of podocyte uPAR is inhibited by amiloride treatment. Therefore, we detected the activity of β3 integrin in podocytes by flow cytometry for active β3 integrin and immunofluorescent double labeling of synaptopodin and active β3 integrin. We labeled β3 integrin with AP 5 antibody, which is known to recognize active β3 integrin [8, 35].

As expected, a strong induction of Ap 5 labeling was observed in podocytes treated with LPS or in animals, including NTX rats and mice treated with LPS (Figure 6). In contrast, podocyte Ap 5 labeling was reduced when treated with amiloride, but not triamterene (Figure 6A–C), suggesting that amiloride inhibits activation of β3 integrin in podocytes, which may be a mechanism for amiloride’s antiproteinuric effect, given recent report that the activation of β3 integrin mediates uPAR-induced cellular events leading to proteinuria [8, 9].

Discussion

The current report suggests that amiloride possesses antiproteinuric properties. This antiproteinuric effect of amiloride can be partially attributed to the drug’s inhibition of uPAR expression in kidney podocytes. Specifically, our results demonstrate that in vitro treatment with amiloride inhibits uPAR mRNA and protein synthesis in podocytes, and in vivo treatment with amiloride reduces proteinuria in animal models of kidney disease, including the 5/6 nephrectomy rat FSGS model and the LPS mouse model of transient proteinuria.
Proteinuria is a major health care problem that affects several 100 million people worldwide. It is a cardinal sign and a prognostic marker of kidney disease and also an independent risk factor for cardiovascular morbidity and mortality [36]. Therefore, the reduction or prevention of proteinuria is highly desirable. Although the glomerular endothelium, GBM and podocytes all contribute to the filtration barrier, the podocytes seem to be the most critical part of the filtration unit [14]. Pathogenic pathways activated in podocytes during proteinuria have been identified, and these findings pinpoint the podocyte as the most obvious candidate for therapeutic intervention [13, 37]. Of particular relevance to the present study is uPAR-β3 integrin signaling, but not urokinase, in the development of proteinuria [8, 9]. uPAR is a proteinase receptor and is also involved in non-proteolytic pathways, mainly through interactions with other plasma membrane proteins such as integrins, caveolin and G-protein-coupled receptors [6]. uPAR, together with β3 integrin and vitronectin, mediates podocyte dysfunction and development of proteinuria in mice. It has been reported that uPAR-deficient mice (Plaur−/− mice) were protected from proteinuria in response to LPS and most notably, when uPAR was reconstituted, Plaur−/− mice developed heavy proteinuria after LPS injection. This study shows a physiological role for uPAR signaling in the development of proteinuria [8].

![Fig. 5. Amiloride treatment inhibits uPAR induction in podocytes in vivo.](image)

(A) Double immunofluorescence staining for uPAR (red) and synaptopodin (synpo, green), a podocyte marker, in glomeruli from sham rats or NTX rats treated with vehicle, amiloride or triamterene. Podocytes of vehicle-treated NTX rats showed an increased expression of uPAR protein. Amiloride treatment substantially inhibits uPAR induction. In contrast, treatment with triamterene failed to inhibit uPAR. (B) Same as in A, but now in the LPS mice. Treatment with LPS substantially enhanced podocyte uPAR expression. Treatment with amiloride, but not triamterene, significantly inhibited uPAR induction. (C) Quantitative real-time RT–PCR was performed on kidney cortex isolated from NTX rats. PLAUR mRNA was upregulated in vehicle-treated NTX rats. Treatment with amiloride, but not triamterene, inhibited PLAUR mRNA expression in the kidney cortex. (D) Same as in C, but now in the LPS mice. Vehicle-treated LPS mice showed an increased PLAUR mRNA expression. Treatment with amiloride, but not triamterene, inhibited PLAUR mRNA expression in the kidney cortex. All values are expressed as means ± SD. **P < 0.01 versus untreated NTX rats or untreated LPS mice; ###P < 0.01 versus sham rats or control mice.

Proteinuria is a major health care problem that affects several 100 million people worldwide. It is a cardinal sign and a prognostic marker of kidney disease and also an independent risk factor for cardiovascular morbidity and mortality [36]. Therefore, the reduction or prevention of proteinuria is highly desirable. Although the glomerular endothelium, GBM and podocytes all contribute to the filtration barrier, the podocytes seem to be the most critical part of the filtration unit [14]. Pathogenic pathways activated in podocytes during proteinuria have been identified, and these findings pinpoint the podocyte as the most obvious candidate for therapeutic intervention [13, 37]. Of particular relevance to the present study is uPAR-β3 integrin signaling, but not urokinase, in the development of proteinuria [8, 9]. uPAR is a proteinase receptor and is also involved in non-proteolytic pathways, mainly through interactions with other plasma membrane proteins such as integrins, caveolin and G-protein-coupled receptors [6]. uPAR, together with β3 integrin and vitronectin, mediates podocyte dysfunction and development of proteinuria in mice. It has been reported that uPAR-deficient mice (Plaur−/− mice) were protected from proteinuria in response to LPS and most notably, when uPAR was reconstituted, Plaur−/− mice developed heavy proteinuria after LPS injection. This study shows a physiological role for uPAR signaling in the development of proteinuria [8]. In addition, the soluble form of uPAR has just now been described as a circulating factor in FSGS [9]. Using two models for podocyte-mediated proteinuria (5/6 nephrectomy rat model and the LPS mouse model of transient proteinuria), we describe an increased expression of uPAR leading to the activation of β3 integrin. Previous studies have shown that amiloride inhibits uPAR expression in tumor-infiltrating lymphocytes [3] and colon cancer cells [4, 5]. In the present study, we provide a new evidence in support of amiloride’s inhibitory action on uPAR not only in cultured differentiated podocytes treated with LPS but also in animal models of proteinuria. Given these findings,
along with reports of uPAR signaling in the development of proteinuria, we hypothesized that amiloride would prove antiproteinuric. As expected, in these two animal models, treatment with amiloride, but not triamterene, reduced proteinuria. Given the effect of amiloride in our animal models of proteinuria as well as the previously published effect in saline-drinking SHRSP [19], it is reasonable to conclude that amiloride’s antiproteinuric action may be partially related to its inhibition of uPAR. Despite amiloride’s antiproteinuric effect confirmed in our models (LPS mice and NTX rats) and SHRSP, our observations are not completely analogous to those made in a previous study in which, although low-dose amiloride at 1 mg kg day\(^{-1}\) lowered proteinuria in PAN rat model, the reduction of proteinuria was not statistically significant [38]. It is possible that higher doses of amiloride may result in a further reduction in proteinuria.

Given the effectiveness of amiloride in reducing proteinuria, the renoprotective effects were also examined in TNX rat model. Treatment of TNX rats with amiloride reduced glomerulosclerosis, a marker of progressive kidney diseases. These results indicate that amiloride’s renoprotective effect may, in part, be attributable to its antiproteinuric action in TNX rat model.

It has been shown that uPAR interact with several integrins that modulate their ligand-binding activities [10], \(\beta_3\) integrin is among those that are modulated by uPAR, and activation of \(\beta_3\) integrin in glomeruli is decreased in uPAR-deficient mice [10]. The activation of \(\beta_3\) integrin is a key signal that mediates uPAR-induced cellular events leading to proteinuria; the expression of a constitutively active \(\beta_3\) integrin is sufficient to induce proteinuria, even in the absence of uPAR [8]. Interestingly, proteinuria caused by uPAR-\(\beta_3\)-integrin signaling can be prevented and reduced by cycloRGDfV [8], a selective inhibitor of \(\beta_3\) integrin [11] or by plasmapheresis that lowers the amount of soluble uPAR [9].

Our results showed an induction of active \(\beta_3\) integrin in podocytes treated with LPS or in animal models of proteinuria. Treatment with amiloride, but not triamterene, reduced activation of \(\beta_3\) integrin, which may be a mechanism for amiloride’s antiproteinuric effect given recent report that the activation of \(\beta_3\) integrin mediates uPAR-induced cellular events leading to proteinuria [8].

Most cases of proteinuria are associated with the effacement (retraction) of podocyte foot processes, which represent podocyte dynamics in vivo or motility of podocyte [14, 25].
There, podocytes stay attached to the GBM, but changes in altered podocyte foot process dynamics result in foot process effacement and proteinuria. In some forms of inflammatory glomerular diseases, such as crescentic glomerulonephritis, podocytes can move out of their microenvironment into areas of crescentic glomerular damage [15, 16]. Interestingly, it has also been reported that amiloride completely prevented pulmonary metastases when given to rats in their drinking water [4, 5]. This amiloride-induced antimetastatic action was reported to be linked to its inhibitory effects on uPAR expression [7, 17, 18]. In the present study, uPAR, a molecule associated with cell motility, is highly expressed on cell surface of diseased podocytes. In contrast, there is little expression on normal quiescent podocytes. To better understand the drug’s inhibitory effect on uPAR expression, we examined motility of podocytes treated with amiloride. Our data showed that amiloride reduced podocyte-directed motility and random migration.

Given the effect of amiloride, but not triamterene, in our animal models of proteinuria as well as the previously published effects in models of SHRSP [19], it is reasonable to predict its efficacy in kidney diseases characterized by podocyte dysfunction and uPAR induction in podocytes.

In conclusion, we provide here the experimental basis for a therapeutic benefit of amiloride in podocyte dysfunction or proteinuria that can readily be used in the clinics as adjunct therapy with other anti-proteinuric modalities.

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References


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