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Research Article

Amiloride lowers blood pressure and attenuates urine plasminogen activation in patients with treatment–resistant hypertension

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Abstract

In conditions with albuminuria, plasminogen is aberrantly filtered across the glomerular barrier and activated along the tubular system to plasmin. In the collecting duct, plasmin activates epithelial sodium channels (ENaC) proteolytically. Hyperactivity of ENaC could link microalbuminuria/proteinuria to resistant hypertension. Amiloride, an ENaC inhibitor, inhibits urokinase–type plasminogen activator. We hypothesized that amiloride (1) reduces blood pressure (BP); (2) attenuates plasminogen–to–plasmin activation; and (3) inhibits urine urokinase–type plasminogen activator in patients with resistant hypertension and type 2 diabetes mellitus (T2DM).In an open–label, non–randomized, 8–week intervention study, a cohort (n = 80) of patients with resistant hypertension and T2DM were included. Amiloride (5 mg/d) was added to previous triple antihypertensive treatment (including a diuretic and an inhibitor of the renin–angiotensin–aldosterone system) and increased to 10 mg if BP control was not achieved at 4 weeks. Complete dataset for urine analysis was available in 60 patients. Systolic and diastolic BP measured by ambulatory BP monitoring and office monitoring were significantly reduced. Average daytime BP was reduced by 6.3/3.0 mm Hg. Seven of 80 cases (9%) discontinued amiloride due to hyperkalemia >5.5 mol/L, the most frequent adverse event. Urinary plasmin(ogen) and albumin excretions were significantly reduced after amiloride treatment ($P < .0001$). Urokinase activity was detectable in macroalbuminuric urine, with a tendency toward reduction in activity after amiloride treatment. Amiloride lowers BP, urine plasminogen excretion and activation, and albumin/creatinine ratio, and is a relevant add–on medication for the treatment of resistant hypertension in patients with T2DM and microalbuminuria. J Am Soc Hypertens 2014;8(12):872–881.

Keywords: Albuminuria; epithelial sodium channel; plasmin; type 2 diabetes mellitus; urinary plasminogen activator.

Introduction

Hypertension increases the risk of cardiovascular and renal disease.[1] Type 2 diabetes mellitus (T2DM) augments this risk.[2,3] A major predictor of increased cardiovascular morbidity and mortality is albuminuria.[4] Patients with T2DM often develop microalbuminuria and hypertension, which is a clinical challenge to treat with respect to achievement of accepted blood pressure (BP) goals. A subset of these patients can be defined with treatment–resistant hypertension in whom BP remains above goal despite the

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use of three or more antihypertensive medications in effective doses including a diuretic.[5] Volume overload is a characteristic finding in these patients, which indicates the use of diuretics, but insight in causal mechanisms in this particular group is limited.[6] Recent studies have shown that aberrant filtration of plasma proteases, in particular plasminogen, may activate the epithelial sodium channel (ENaC) by proteolytic cleavage of the extracellular domain of the gamma subunit.[7] This interaction introduces a mechanistic concept for the coupling between microalbuminuria, hypertension, and the salt–sensitivity of BP in these patients.[8,9] However, in vivo data supporting the hypothesis are limited. Aberrantly filtered plasminogen in pre–urine is likely to be activated by the urokinase–type plasminogen activator (uPA), which is present in urine, and secreted by the tubular epithelium.[10] In proteinuria, both plasminogen and active plasmin can be detected in urine.[8] It was hypothesized that difficult–to–treat hypertension in T2DM patients with concurrent micro– or macroalbuminuria predicts sensitivity to the direct ENaC inhibitor amiloride despite use of several other classes of drugs. A previous study supports that amiloride may provide additional therapeutic benefit when added to another diuretic and a calcium antagonist in hypertensive African Americans, an ethnic group that often displays difficult–to–treat hypertension.[11] Of note, amiloride is an established, ''off–target'' competitive inhibitor of uPA (Ki $\sim$7 μmol/L).[12] Concentrations of amiloride $\sim$20 μmol/L have been reported in urine.[13] To our knowledge, it is not known whether uPA is present in increased amounts in urine from patients with microalbuminuria, and whether amiloride, in therapeutic concentrations, inhibits uPA activity in human urine. We therefore hypothesized additionally, that urine uPA is a target for amiloride in patients with hypertension and micro– or macroalbuminuria with aberrant presence of plasminogen. The hypotheses were tested in an open–label, non–randomized, intervention study. Amiloride was administered as an add–on to triple antihypertensive treatment in patients with treatment resistant hypertension and T2DM. Patients were recruited following a wash–out period after participation in a previously published study on the antihypertensive effect of spironolactone and included again.[14] Plasmin(ogen) was present in urine from patients with resistant hypertension and T2DM[14] in amounts that correlated with albumin, and it was hypothesized that the antihypertensive effect of amiloride would correlate directly with the degree of plasminogen and albumin in urine and thus the putative degree of proteolytic ENaC activation.[15]

Methods

Study Design and Material

After participation in a randomized double–blinded placebo–controlled trial of the effect of spironolactone versus placebo in type 2 diabetic patients with resistant hypertension,[14] patients were invited to participate in the present observational open–label extension study. Patients with T2DM, aged between 30 and 75 years and with therapy–resistant hypertension defined as BP $\geq$130/80 mm Hg measured as daytime average by ambulatory BP monitoring (ABPM) despite treatment with three or more antihypertensive drugs of different classes, including a diuretic in optimal doses, were included. Detailed inclusion and exclusion criteria are given.[14]

A minimum of 2 weeks wash–out period (median, 28 days; range, 14–118 days) took place between the previous spironolactone study[14] and inclusion in the present study on amiloride to minimize potential carry–over effects while subjects continued triple antihypertensive medication.

ABPM and morning spot urine sample were performed at baseline and at follow–up at 8 weeks. Amiloride (5 mg daily) was added to the patients' previous triple antihypertensive treatment. Previous antihypertensive treatment remained unchanged during trial. A validated device (TM2430, A and D Company Ltd. Tokyo, Japan),[16] measuring BP at 15–minute intervals during the day (07.00 h–22.00 h) and at 30–minute intervals during the night (22.00 h–07.00 h), was used to measure ABPM. Follow–up visits were scheduled after 4 and 8 weeks.

Office BP was measured at baseline and at scheduled visits. Office BP was recorded three times per session by a validated automatic oscillometric device (UA 767, A&D Company Ltd, Tokyo, Japan)[17] in the sitting position; the average of the second and third measurements were recorded. If office BP had not reached target <130/80 mm Hg after 4 weeks, the dose of amiloride was increased to 10 mg daily. Safety potassium measurements were done 1 week after initiating amiloride treatment and 1 week after dose titration at 4 weeks. If potassium increased to >5.5 mmol/L, amiloride was discontinued. Adherence was determined by counting returned tablets, and patients were considered compliant when taking more than 80% of study medication.

The study protocol was approved by the Ethical Committee of the Region of Southern Denmark, S-20090135, and at the Danish Medicines Agency, EudraCT 2009-017033-22. The study was performed in accordance with the Helsinki Declaration and International Conference on Harmonization–Good Clinical Practice rules. Good Clinical Practice monitoring was done by the Department of Clinical Pharmacology, Odense University Hospital. The study was registered at clincalTrials.gov as NCT02122731.

Urine creatinine and albumin concentration were analyzed immediately by standard methods at the department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark. Remaining aliquots of the spot urine samples were stored at −80°C in 4.5 mL Nunc CryoTubes.

Urinary plasminogen concentration was analyzed by a commercial kit (Human Plasminogen Total Antigen Assay [Cat# IHPLGKT-TOT, Innovative Research, Novi, MI]).

Urinary urokinase-type plasminogen activator (uPA) activity was analyzed by a commercial activity assay kit (Chemicon International [Cat# ECM600]). Before analysis, urine was concentrated in Amicon tubes.

Western immunoblotting (WB) was performed on baseline and follow–up urine samples from all of the patients in the cohort with macroalbuminuria (n = 11) and from two randomly selected with normoalbuminuria and 13 with microalbuminuria and compared for abundance of plasmin. Concentrated urine was mixed with NuPAGE Sample Reducing Agent (10×; Invitrogen, Carlsbad, CA), NuPAGE LDS Sample Buffer (4×; Invitrogen), and heat denatured. Samples were run on a Ready Gel 4%–12% Tris–HCl (Novex Lifetechnologies), and subsequently blotted onto an Immobilon polyvinylidene difluoridemembrane (Millipore, Immobilon-P transfer membrane, pore size 0.45 μm, Millipore Corporation, Bedford, MA). The polyclonal goat anti–human plasminogen ab 6189-100 (Abcam) was used as primary antibody, and HRP–conjugated anti–goat (DakoCytomation, Glostrup, Denmark) was used as secondary antibody. Blots were developed using Western Lighting ECL pro, Perkin Elmer, Inc. The monoclonal mouse Anti–Urokinase antibody ab8473 (Abcam, Cambridge UK) was used as primary antibody, and HRP–conjugated antimouse (DakoCytomation, Glostrup, Denmark) as secondary antibody for uPA WB.

Abundance of urinary plasmin and uPA before and after intervention was quantified by scanning densitometry analysis (Quantity One version 4.6.3, Gel Doc 2000, Bio Rad).

Statistical Analyses

All data were analyzed per protocol, meaning only complete sets of data were used for analysis.

Intention–to–treat analysis was also used on ambulatory blood pressure variables with the principle of last–observation–carried–forward. In order to detect a minimal difference of 7 mm Hg standard deviation (SD), 16 mm Hg, sample size calculation showed a necessary minimum of 55 participants to detect this difference and provide the study with 90% power ($P < .05$). Assuming a dropout rate of 15%, we aimed to include 65 subjects. All data were tested for normal distribution and presented as mean and standard deviation/standard error of the mean. Categorical variables were described by the number of cases and the percentages of categories. In case of non–normal distribution, data were either presented as median or log–transformed. For normally distributed data, the effect of intervention was analyzed by the paired *t*–test. If data were not normally distributed, the nonparametric *t*–test (Wilcoxon) and nonparametric correlation (Spearman) were used for statistical analysis.

STATA 11 (StataCorp) statistical software was used for all data analysis. GraphPad Prism 5 (GraphPad Software, San Diego, CA) was used for generating figures.

P values <.05 were considered as statistically significant.

Results

Study Parameters

Eighty subjects with resistant hypertension and T2DM were included in the present extension study on amiloride (trial profile, Supplemental Figure 1; see online data supplement). Sixty-nine completed the study. Seven participants discontinued the study medication due to hyperkalemia, which was the most frequent adverse event (7/80). Other causes of study discontinuation were severe adverse events: one case of upper gastrointestinal bleeding, one case with severe intestinal infection, one case with severe abdominal pain, and one with symptomatic hypotension (below 110/60 mm Hg). Complete paired dataset for analysis was available on 60 participants. (six only had one urine sample, two only had one ABPM, and one was not compliant). At the end of the study, 43% (26/60) of patients received 5 mg, and 56% (34/60) received 10 mg of amiloride daily. The mean dose was 7.8 mg (SD, 2.5 mg).

In a sub–analysis, we compared the effects of amiloride versus spironolactone in the group (n = 26) of patients who completed and received first spironolactone in the previous study[14] and then amiloride in the present study. Additionally, we found no differences in the effect of amiloride on BT depending on the initial allocation to treatment groups (spironolactone/placebo) in the previous study or differences in baseline BP.[14]

Clinical Characteristics

Of the patients who had a complete dataset (n = 60), median duration of T2DM was 10 years and of hypertension 11 years. Only 7% had normal body mass index (BMI) below 25 kg/m^2; 30% were overweight (BMI, 26–30 kg/m^2) and the remaining 63% were obese, with BMI >30 kg/m^2. Baseline clinical characteristics are presented in Table 1. Of note, median estimated glomular filtration rate (eGFR) was normal, and plasma Na+ and K+ were normal. At baseline, 58% of patients used three antihypertensive drugs, 37% used four, and 5% used more than four. At baseline, 43% (n = 26) had normoalbumiuria (albumin/creatinine ratio [ACR] <30 mg/g), 38% (n = 23) had microalbuminuria (30 mg/g <ACR >300 mg/g), and 18% had macroalbuminuria (n = 11; ACR >300 mg/g). The baseline antihypertensive medication is outlined in Supplemental Table 1 (available online only).

Table 1
Baseline characteristics

Characteristics	Values n = 60
Age (y)	63.2 (61.5–64.8)
Gender (female)	15/60 (25%)
Weight (kilograms)	109.8 (86.7–139.1)
BMI (kg/m^2)	32.5 (25; 42.5)
Abdominal circumference (cm)	112.6 (109.0–116.4)
Metabolic status	
HbA1c	7.3 (7.0–7.6)
Kidney function	
Creatinine (μmol/l)	82 (17.6)
eGFR (ml/min/1.73m2)	81.5 (16.6)
Urinary albumin excretion (mg/g)	35.7 (4.5–639.5)
Vascular variable	
24–hour SBP (mm Hg)	141.1 (10.4)
24–hour DBP (mm Hg)	77.3 (7.1)
24–hour pulse (beat/min)	73.5 (11.6)
Daytime SBP (mm Hg)	143.9 (10.5)
Daytime DBP (mm Hg)	79.1 (7.2)
Daytime pulse (beat/min)	74.6 (11.8)
Nighttime SBP (mm Hg)	126.8 (16.9)
Nighttime DBP (mm Hg)	67.9 (9.6)
Nighttime pulse (beat/min)	67.6 (11.0)
Office SBP (mm Hg)	143.1 (15.3)
Office DBP (mm Hg)	77.7 (9.0)
Office pulse (mm Hg)	71.8 (12.9)
Electrolytes	
Potassium (mmol/L)	3.9 (3.6–4.3)
Sodium (mmol/L)	139.6 (2.4)

BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomular filtration rate; SBP, systolic blood pressure.

Blood pressure data was normally distributed and expressed as mean and standard deviation.

Non-Gaussian distributed data are presented as medians and 5%–95% percentile.

Effect of Amiloride on BP

Mean daytime BP was reduced significantly by 6.0/3.7 ($P < .001/P < .0001$) mm Hg during treatment with amiloride. Also, nighttime BP and 24–hour BP were reduced significantly by 7.1/2.8 mm Hg ($P < .001/P = .004$) and 6.1/3.6 mm Hg ($P < .0001/P < .0001$), respectively (Figure 1A–C). Office BP was reduced by 6.1/2.6 mm Hg ($P = .002/P = .045$). By intention–to–treat analysis, daytime and nighttime BP were reduced significantly by 5.3/3 ($P < .0001/P < .0001$) mm Hg and 6.7/2.6 mm Hg ($P < .0001/P = .0029$), respectively, whereas 24–hour BP was reduced significantly by 5.4/3 mm Hg ($P < .0001/P < .0001$), respectively. In the sub–analysis of 26 patients who also completed the prior placebo–controlled spironolactone study,[14] the effect of the addition of spironolactone on daytime BP was a reduction of 9.5/5.2 mm Hg versus 6.8/5.2 mm Hg when amiloride was added. There were no significant differences in BP reduction between the two drugs ($P = .17/P = .11$).

The reduction in daytime SBP during amiloride treatment was numerically greater for patients with macroalbuminuria (7.8 [SD, 8.3] mm Hg; n = 11) as compared with patients with normoalbuminuria (6.4 [SD, 9.7] mm Hg; n = 26) and microalbuminuria (4.7 [SD, 10.5] mm Hg; n = 23), but not statistically significant.

Effect of Amiloride on Urinary uPA

By WB, uPA was detected in the concentrated urine samples in the patients with macroalbuminuria (n = 8; Figure 2A). Based on the WB, uPA protein abundance in creatinine–normalized urine samples tended to decrease after amiloride treatment, and the decline was significant when measured by densitometry (n = 8; Figure 2B).

Urine uPA activity reached detection limit by chromogenic substrate activity assay in concentrated urine samples from macroalbuminuric patients only. There was a tendency toward reduction in uPA activity in response to amiloride treatment, but it was not significant (Figure 2C). Baseline uPA activity did not correlate to baseline ACR. Delta uPA and delta ACR correlated significantly ($r = 0.42$; $P = .0478$) in patients with micro– or macroalbuminuria (n = 11).

To test whether the observed changes were specific or related to improved filtration barrier, the effect of amiloride on urine albumin excretion was measured, and it was significantly reduced ($P < .0001$) (Figure 2D). Twenty–six patients were normoalbuminuric at baseline, whereas 38 patients displayed normoalbuminuria at follow–up; the change was not statistically significant ($P = .225$).

In the sub–analysis, comparing effects of spironolactone versus amiloride (n = 26), the median reduction in urinary–ACR was not statistically different when amiloride was added compared with spironolactone (5 [83; 237] mg/g vs. 17 [5; 1093] mg/g; $P = .19$).

Effect of Amiloride on urinary plasminogen activation

To obtain an indirect measure of uPA activity, plasminogen/plasmin excretion was examined by WB of urine samples with an antibody that discriminates between zymogen and active plasmin. Validation immunoblotting showed that the antibody detected proteins in a concentrated urine sample from a T2DM patient with proteinuria that co–migrated with a pure human plasmin control and co–migrated with intact plasminogen in serially diluted control human plasma (Figure 3A left). Use of secondary antibody only on the same urine and plasma samples yielded no signal on prolonged exposure (Figure 3A right).

Immunoblotting of subsets of urine samples from n = 13 patients with microalbuminuria and n = 11 with macroalbuminuria (Figure 3 B–E) showed decline in active plasmin

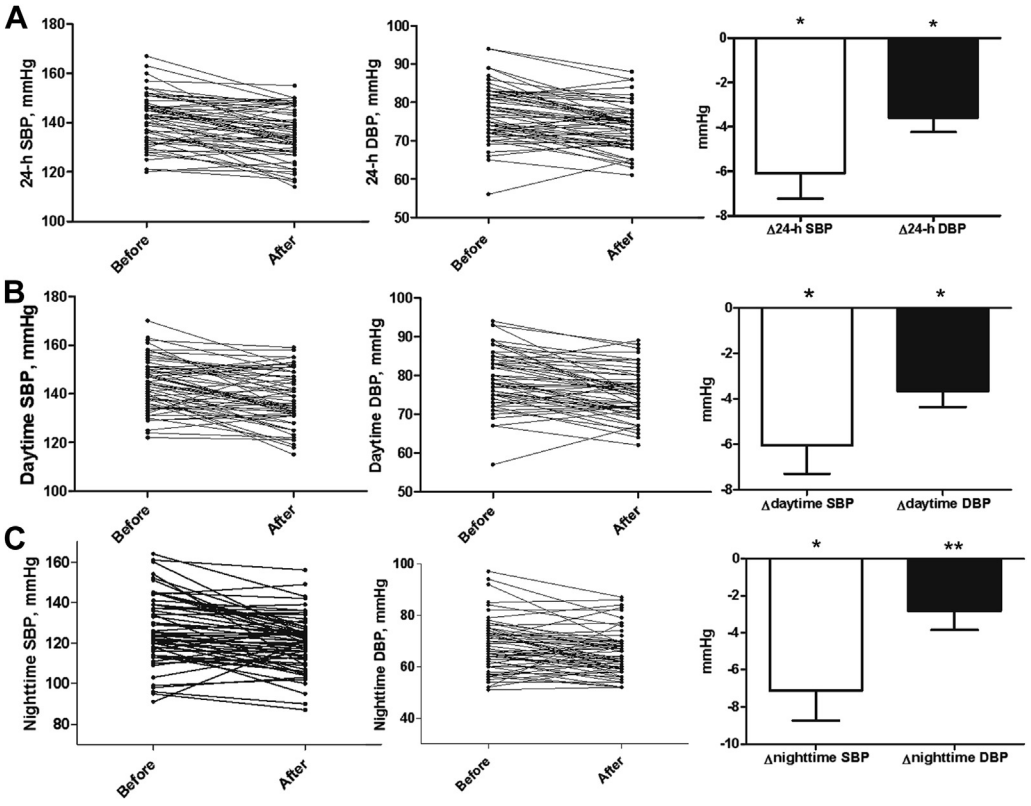

Figure 1. Blood pressures before and after amiloride treatment. The left and middle diagrams show values for systolic (SBP) and diastolic (DBP) blood pressure for each single patient: (A) Ambulatory 24–hour SBP; (B) Daytime SBP; and (C) Nighttime SBP. Columns represent the change (Δ) in blood pressure in response to amiloride, mean ± standard error of the mean (SEM). *$P < .0001$; **$P < .05$.

as measured by densitometry (Figure 3F). For comparison, n = 2 control samples with normal albumin excretion was run in parallel and showed no to faint plasmin signal (Figure 3E, right lanes).

Effect of Amiloride on Total Immunogenic Urine Plasminogen Excretion

Detection of total immunogenic plasmin(ogen) by sensitive enzyme-linked immunosorbent assay in urine before and after amiloride showed no statistically significant change for the entire population. However, because 26 of the 60 samples with normal albumin excretion were below detection limit, the analysis was performed only on albuminuric patients. In these patients (n = 34), urine plasminogen/creatinine ratio displayed a significant reduction ($P = .0306$; Figure 4 A–B).

At baseline, urine plasminogen/urine creatinine ratio (u–plg/u–crea) correlated to all measures of systolic BP measured by ABPM (Figure 4C). U-plg/u-crea was not correlated to the reduction in BP during amiloride treatment, but the reduction in u-plg/u-crea correlated to the reduction in office systolic BP (r = 0.43; $P = .007$) and daytime systolic BP (r = 0.27; $P = .0395$).

Effect of Amiloride Treatment on Plasma Electrolytes and Kidney Function

Plasma potassium concentration increased significantly by 0.5 (SD, 0.5) mmol/L ($P < .0001$) during amiloride treatment. In two out of the 60 cases, hyperkalemia (6.2 mmol/L and 5.8 mmol/L) was discovered in blood samples obtained at the day of study end after 8 weeks of amiloride treatment. Plasma sodium concentration decreased by 1.2 (SD, 2.7) mmol/L ($P = .001$). Also eGFR as estimated by the Chronic Disease Epidemiology Collaboration formula (CKD-EPI) decreased significantly by 5.4 (SD, 10) mL/min ($P < .0001$). Urinary–Na/K remained unchanged (Supplemental Figure 2, available online only). Plasma potassium concentration increased by 0.5 (SD, 0.6) mmol/L when amiloride was added and 0.2 (SD, 0.4) mmol/L when spironolactone was added and compared in the subanalysis of 26 patients. The difference was borderline significant at $P = .0581$.

Discussion

The present study shows a significant BP–lowering effect of amiloride added to triple antihypertensive treatment in

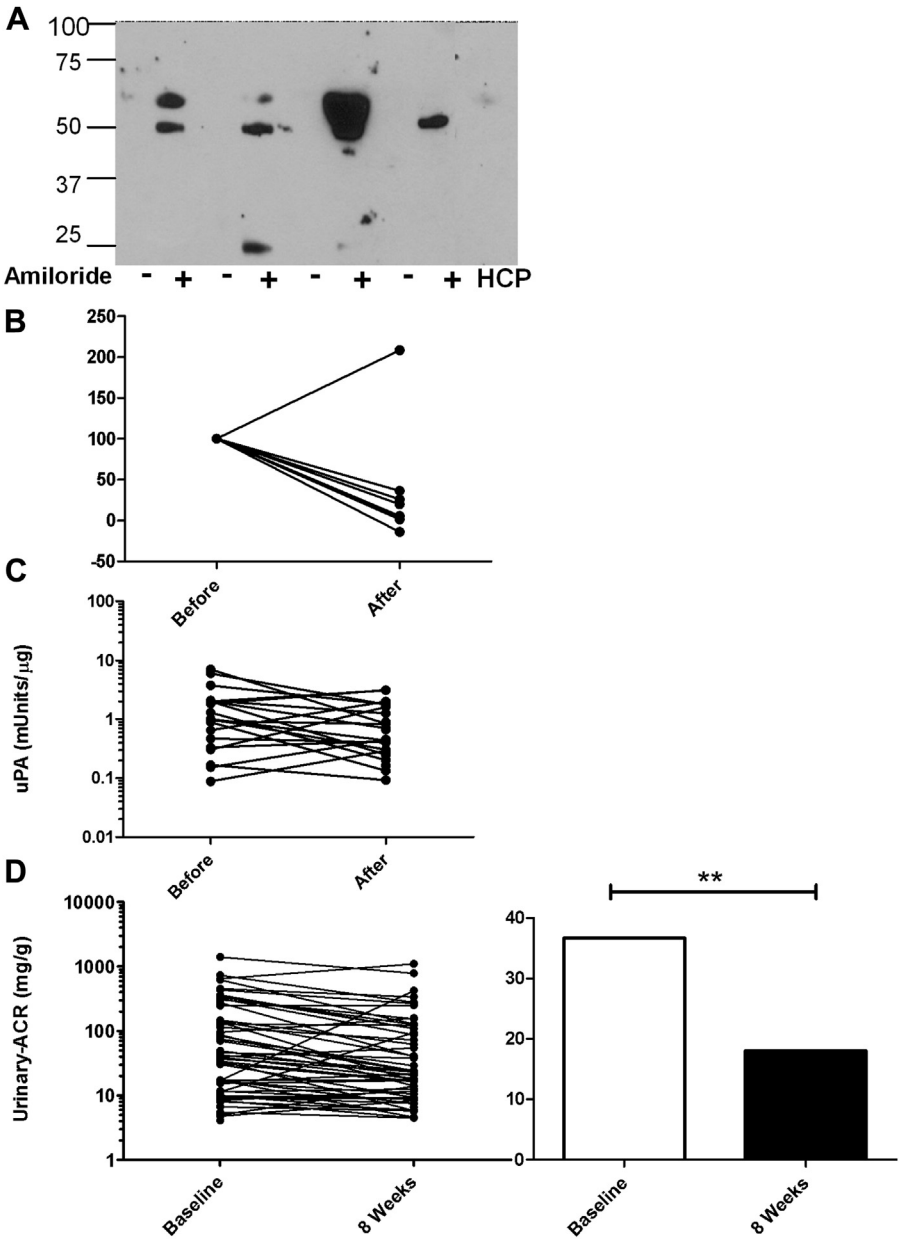

Figure 2. (A) Western immunoblotting for urokinase–type plasminogen activator (uPA) in creatinine–normalized urine samples from four patients before (−) and after (+) amiloride. Urine samples were from patients with highest albumin excretion and concentrated before analysis. Film was exposed for 5 minutes. (B) Densitometric evaluation of the immunoblots for uPA in urine from n = 7 macroalbuminuric patients where the blots showed detectable signal. Seven of eight patients showed a decrease in uPA immunoreactive protein. Expected size 55 kDa (pre–uPA) and 52 kDa HMW uPA. (C) Urine uPA activity was detected by chromogenic assay in urine samples from patients with macroalbuminuria before and after amiloride treatment. There was no significant change. (D) Change in urine albumin excretion with amiloride treatment. Left shows before and after for each patient and columns show medians $^{**}P < .0001$.

patients with resistant hypertension and T2DM. Amiloride lowered urine albumin, plasminogen, plasmin, and uPA excretion, while plasma potassium increased and sodium decreased. A direct inhibitory effect of amiloride on urine uPA activity could not be defined clearly because of the overall decline in urine protein excretion, although the urine level of active plasmin decreased in response to amiloride. U–plasminogen correlated to BP. Targeting ENaC and potentially uPA–plasminogen with amiloride as an add–on appears attractive to achieve BP control in treatment–resistant hypertensive patients.

The recent discovery of proteolytic activation of ENaC[8] and aberrant presence of serine proteases, notably plasmin with ability to activate ENaC,[15] in urine from patients with microalbuminuria/proteinuria, provided a new rationale for specifically targeting ENaC. Aberrant activation of ENaC in vitro by urine from the present patients[15] is compatible with the significant reduction in all measures of systolic and diastolic BP when amiloride was added to the three–drug regimen in this study population. In a recent Cochrane review, the few studies of low–dose amiloride for essential hypertension showed no effect of amiloride as a second drug, which underlines the significance of the present findings.[18] Additional BP reduction with amiloride in this study supports the hypothesis that ENaC overactivity may be a part of the pathophysiology of volume overload in patients with difficult–to–treat hypertension with concomitant albuminuria.[19] With amiloride, 14% of patients

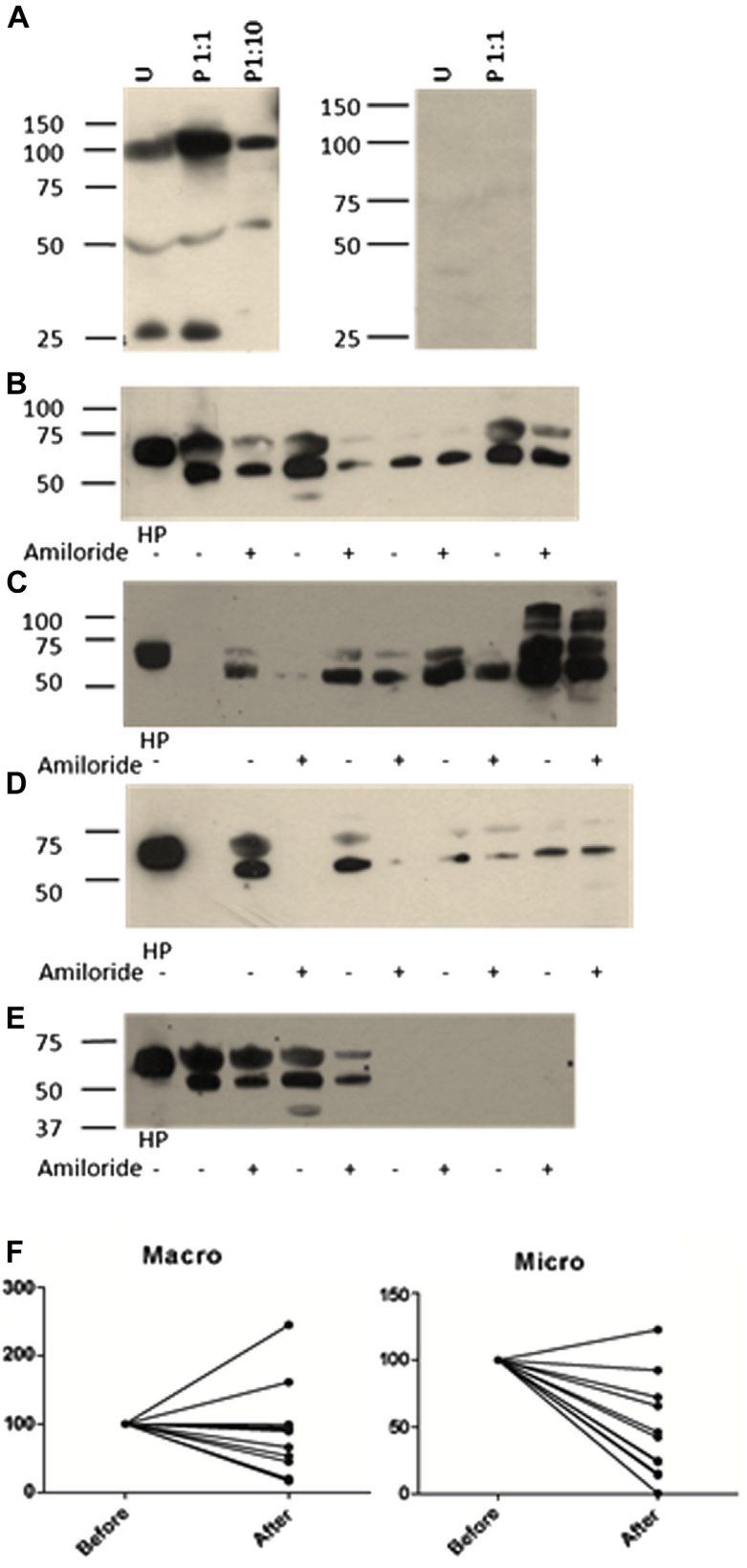

Figure 3. Western immunoblotting for plasminogen–plasmin in urine samples from patients with variable degrees of albumin excretion in urine. (A) Left: Validation of anti–plasminogen antibody with urine (U) from the patient with highest albumin excretion in the cohort (1400 mg/g) compared with blood plasma from a control person undiluted (P 1:1) and diluted 1:10 (P1:10), film exposed for 5 seconds. The right blot shows a negative control with the same urine and plasma sample as in the left blot, but with no primary antibody. Exposure was for 3 minutes. Predicted molecular sizes for plasmin(ogen) ~90 kDa, ~80 kDa, 60 kDa, and 25 kDa. (B–E) Western immunblotting for plasminogen–plasmin in paired urine samples from patients without (−) and with amiloride (+) treatment and (B) macroalbuminuria; (C) microalbuminuria; (D) microalbuminauria; and (E) macroalbuminuria in the first four lanes and normal albumin excretion in the last four lanes. ''HP'' is a positive control where pure human active plasmin (1 μg) was run in parallel. All films were exposed ~1 minute. (F) When splasmin signal by densitometry in the ''before'' amiloride sample was set to 100%, there was a significant decline after amiloride in the microalbuminuria group, whereas this was not significant in the macroalbuminuria group.

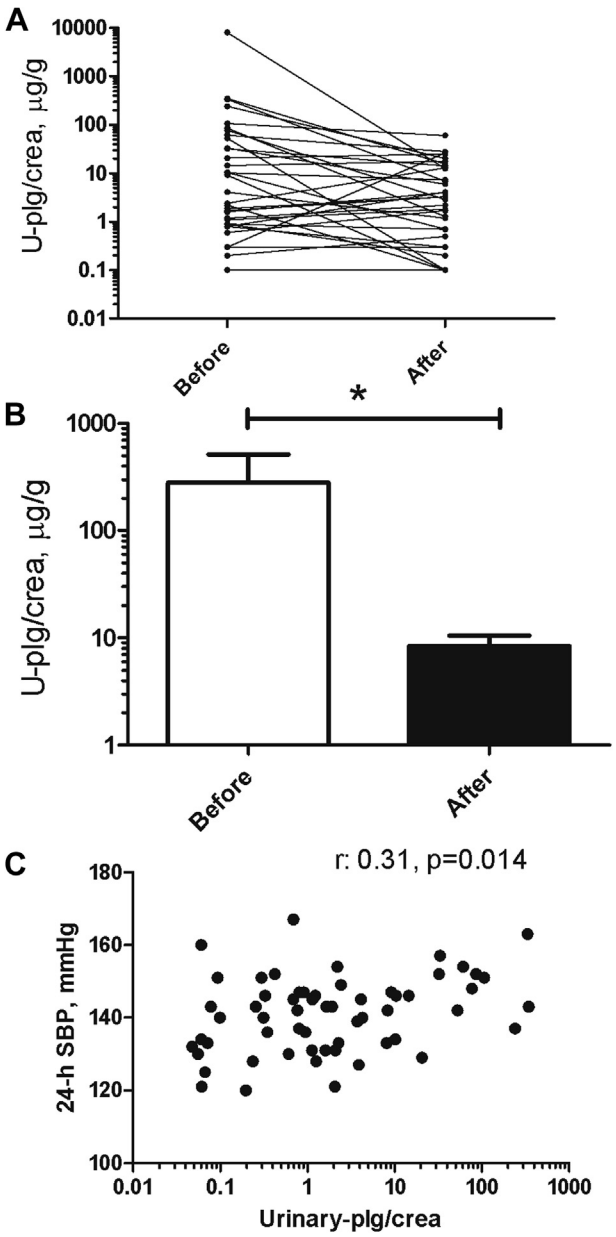

Figure 4. (A–B) Change in immunodetectable total urine plasminogen (plasminogen, plasmin, and plasmin–antiplasmin complexes and fragments) by enzyme-linked immunosorbent assay of spot urine samples from each albuminuric patient before and after amiloride ($P = .03$). (A) Only albuminuric samples where plasminogen was above detection limit are shown (n = 34 of 60). (B) The graph displays mean total plasminogen/creatinine ratio in spot urine before and after amiloride ± standard error of the means (SEM). *$P < .05$. (C) The graph displays the association between 24–hour systolic blood pressure (SBP) and urinary plasminogen excretion before amiloride treatment.

reached target office BP of <130/80 mm Hg, and with recent guidelines, 40% (24/60) reached target office <140/85 mm Hg for diabetic patients.[20] These effects are in agreement with a previous study, where 10 mg amiloride was added to a multidrug regimen including a renin–angiotensin–aldosterone system–blocking agent in patients with resistant hypertension.[21] Similar BP reductions (9.8/3.4 mm Hg) were found in a placebo–controlled study by Saha et al in African Americans, where 10 mg amiloride was added to another diuretic and a calcium antagonist.[11] Due to secondary increase in plasma aldosterone after amiloride treatment, the combination of spironolactone and amiloride was also assessed in this study; the combination of both drugs was superior compared with treatment with either alone.[11] The effect on albumin excretion was not reported in that study.[11] In our sub–analysis of 26 resistant hypertensive patients with T2DM receiving first spironolactone and then amiloride, the effect of each single drug on BP was not statistically different. In the present study, BP reductions were obtained with lower doses of amiloride (7.8 mg daily) as compared with 10 mg daily in the two previously mentioned studies. The optimal dose–response of amiloride is not established. Most trials investigated doses of 2.5–10 mg amiloride daily typically in combination with hydrochlorthiazide. Whether better BP reduction would have been achieved with higher doses, for example, 20 mg of amiloride as suggested by Lane et al, is not known.[22] The present study was open–label, and the absence of a placebo–control group is a clear limitation. On the other hand, in the same patients, no effect of placebo treatment was observed in the months preceding the present add–on study.[14] A significant risk of severe hyperkalemia in patients with diabetes and renal insufficiency and on angiotensin–converting–enzyme inhibitors/angiotensin receptor blockers has been described.[23] Hyperkalemia was indeed the most frequent adverse event, and amiloride was discontinued due to a serum–potassium >5.5 mmol/L in seven of 80 patients. Hyperkalemia developed typically in patients with eGFR in the lower range (CKD–EPI, 36–82 mL/min/1.73 m^3), and there was just one case of severe hyperkalemia (6.2 mmol/L) discovered at the last visit, indicating a protracted effect. Important limitations are the exclusive white ethnicity, male predominance, and the large proportion of well–controlled type 2 diabetics with only a small fraction with albuminuria. The only randomized, placebo–controlled study that tested amiloride addition was from African Americans. Here, the patients benefitted significantly with respect to BP control.[11] Addition of amiloride significantly reduced albuminuria in the present cohort. It was shown recently, in animal models, that amiloride ameliorated podocyte dysfunction and reduced proteinuria in rats.[24] Whether the reduction in albuminuria by amiloride is the result of specific podocyte protection or due to the significant BP reduction remains unanswered. Similar BP reductions along with reduction in albuminuria were also observed with the addition of spironolactone in the same patients.[14] The initial reduction in eGFR occurs frequently with BP–lowering drugs, including

angiotensin–converting–enzyme inhibitors/angiotensin receptor blockers, and is most likely the result of a reduction in intraglomerular pressure and predicts better long–term function.[25]

Plasminogen is filtered aberrantly in proportion to albumin in the present patients, and their urine activates ENaC current in collecting duct cells in vitro.[15] Urokinase (uPA) is the main plasminogen activator in the urinary system.[26] Intratubular saturation may occur since intact zymogen, plasminogen appeared only in urine samples with macroalbuminuria, while in microalbuminuria, only active plasmin was observed (Figure 3).[7,27] Amiloride is an off–target inhibitor of uPA with a K_i of 7 mmol/L,[12] which is a concentration that is reached in urine.[13] In rats with nephrotic syndrome, amiloride in higher doses (2 mg/kg) attenuated intratubular activation of plasminogen to plasmin without changing overall proteinuria (K.B.B., unpublished data, 2014). The present data showed that urine uPA was detectable only after in vitro concentration of urine samples and in those patients with macroalbuminuria. The molecular weight of pro–uPA is ca.55 kDa, which is not far from albumin, and since uPA also circulates in plasma, the present observations would be compatible with aberrant filtration of uPA, although increased tubular secretion cannot be excluded.[10] There was a tendency that uPA protein decreased along with albumin in response to amiloride, which would be compatible with the notion of aberrant filtration. With these observations, the observed significant decrease in active plasmin and total plasmin(-ogen) in urine could equally well be attributed to attenuated aberrant filtration of uPA/plasminogen as an inhibitory effect of amiloride on uPA. Irrespective of the mechanism, the end effect is likely to be beneficial for the patient since plasmin in urine may disturb not only ENaC but also calcium metabolism.[28] The fact that amiloride exerts its effect from the intratubular luminal side is convenient in the present context. An adverse cycle can be interrupted because active plasmin may activate pro–uPA and vice versa,[29] and plasminogen may be captured at the principal cell apical membrane by prostasin[8,30] or the plasminogen receptor Plg-R_{kt}.[31] In summary, amiloride lowers blood pressure, ACR, and and u-plasminogen activation in albuminuric patients with T2DM and resistant hypertension. Amiloride should be further tested as a relevant add–on medication for treatment of resistant hypertension.

Conclusions

The present data are in accordance with significant and beneficial antihypertensive effect of amiloride as a single, add–on therapy in T2DM patients with treatment–resistant hypertension. In the 8–week intervention period, amiloride significantly lowered albumin excretion and thereby also plasminogen excretion and active plasmin in urine. An off–target effect of amiloride on urokinase could therefore be involved. Amiloride resulted in more than 40% of the patients reaching current treatment goals with hyperkalemia in 7% of patients. Larger, long–term follow–up studies in a placebo–controlled design are, however, needed to evaluate the effect of the addition of amiloride on cardiovascular and renal morbidity and mortality.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jash.2014.09.019.

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