Rostafuroxin Protects from Podocyte Injury and Proteinuria Induced by Adducin Genetic Variants and Ouabain

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ABSTRACT

Glomerulopathies are important causes of morbidity and mortality. Selective therapies that address the underlying mechanisms are still lacking. Recently, two mechanisms, mutant β-adducin and ouabain, have been found to be involved in glomerular podocytopathies and proteinuria through nephrin downregulation. The main purpose of the present study was to investigate whether rostafuroxin, a novel antihypertensive agent developed as a selective inhibitor of Src-SH2 interaction with mutant adducin- and ouabain-activated Na,K-ATPase, may protect podocytes from adducin- and ouabain-induced effects, thus representing a novel pharmacologic approach for the therapy of podocytopathies and proteinuria caused by the aforementioned mechanisms. To study the effect of rostafuroxin on podocyte protein changes and proteinuria, mice carrying mutant β-adducin and ouabain hypertensive rats were orally treated with 100 μg/kg per day rostafuroxin. Primary podocytes from congenic rats carrying mutant α-adducin or β-adducin (NB) from Milan hypertensive rats and normal rat podocytes incubated with 10⁻⁹ M ouabain were cultured with 10⁻⁹ M rostafuroxin. The results indicated that mutant β-adducin and ouabain caused podocyte nephrin loss and proteinuria in animal models. These alterations were reproduced in primary podocytes from NB rats and normal rats incubated with ouabain. Treatment of animals, or incubation of cultured podocytes with rostafuroxin, reverted mutant β-adducin- and ouabain-induced effects on nephrin protein expression and proteinuria. We conclude that rostafuroxin prevented podocyte lesions and proteinuria due to mutant β-adducin and ouabain in animal models. This suggests a potential therapeutic effect of rostafuroxin in patients with glomerular disease progression associated with these two mechanisms.

Introduction

Glomerular diseases leading to progressive kidney failure represent a pivotal cause of morbidity and mortality. Proteinuria is the hallmark of a broad spectrum of podocytopathies characterized by podocyte foot process effacement and alterations of the slit diaphragm, a dynamic signaling hub able to initiate complex signaling networks and integrate multiple intercellular signals via actin cytoskeleton (Grahammer et al., 2013).

Understanding the genetic molecular mechanisms leading to glomerular podocyte injury and proteinuria may help with the identification of potential target molecules and the development of new and specific therapies for the prevention and treatment of glomerular diseases.

Mutations in several podocyte genes have been associated with monogenic and polygenic syndromes that feature proteinuria (Liu et al., 2001; Wiggins, 2007; Mathieson, 2008; Picone and Licht, 2011; Grahammer et al., 2013). In this context, we have contributed to unraveling the role of a polymorphism of the cytoskeletal protein adducin and high levels of endogenous ouabain (EO) in hypertension and glomerular podocyte damage, both in animal models and in patients (Bianchi et al., 1994; Bianchi, 2005; Manunta et al., 2009; Ferrandi et al., 2010a,b). These studies have shown that mutant α-adducin and ouabain activate Src tyrosine kinase and the Src-dependent Na,K-ATPase phosphorylation and its signaling network, associated with an overall increase of renal tubular sodium reabsorption and blood pressure (BP) (Ferrandi et al., 2010a). Rostafuroxin, a novel antihypertensive compound (Quadri et al., 1997), disrupts mutant α-adducin– and ouabain-mediated interactions, favoring the normalization of renal Src-Na,K-ATPase signaling and BP in hypertensive rats (Ferrari et al., 1998, 1999a,b; Ferrandi et al., 2010a) and in patients carrying

ABBR: ABL1, Abelson tyrosine-protein kinase 1; BP, blood pressure; EO, endogenous ouabain; HR, heart rate; KO, β-adducin knockout mice; MHS, Milan hypertensive rats; MNS, Milan normotensive rats; NA, congenic rats for mutant α-adducin from MHS rats; NB, congenic rats for mutant β-adducin from MHS rats; OHR, ouabain hypertensive rats; PST2238, 17β-[3-furyl]-5β-androstan-3β,14β,17α-triol; SBP, systolic blood pressure; WT, wild-type mice; ZO-1, Zona Occludens protein 1.
a specific genetic profile that includes genes encoding for adducin variants and enzymes controlling ouabain synthesis and transport (Lanzani et al., 2010).

Recently, we produced data indicating that mutant β-adducin and EO/ouabain affect the expression of nephrin (Ferrandi et al., 2010b; Bignami et al., 2013), a selective podocyte marker protein (Verma et al., 2006), both in animal models and in patients.

The possible protective effect of rostafuroxin from glomerular injury has not yet been documented. Therefore, the main purpose of the present study was to investigate whether rostafuroxin can counteract mutant β-adducin– and ouabain-mediated effects, being able to prevent podocyte protein loss and the onset of proteinuria. To this aim, we investigated the effect of rostafuroxin on BP, urinary protein excretion, and podocyte protein expression in mice carrying mutant β-adducin, compared with mice with the knockout of β-adducin, and in ouabain hypertensive rats (OHR) obtained by a chronic subcutaneous infusion of a low dose of ouabain. The effect of rostafuroxin on podocyte protein expression has also been investigated in cultured podocytes from congenic rat substrains for α- and β-adducin, obtained by introgressing the mutant α-adducin (NA congenic strain) or β-adducin (NB congenic strain) from the Milan hypertensive rats (MHS) into the Milan normotensive rats (MNS), genetic background, and normal rat podocytes incubated with ouabain.

The results indicated that rostafuroxin exerts a protective effect on podocyte damage induced by mutant β-adducin and ouabain in animal models. In view of the possible translatability of the data from animals to humans, it is reasonable to speculate that the compound may represent a drug of choice for patients.

### Materials and Methods

#### Chemicals

Rostafuroxin (PST2238; 17β-(3-furyl)-5β-androstan-3β,14β,17α-triol) is a digitoxigenin derivative synthesized at Prassis Sigma-Tau Research Institute (Settimo Milanese, Milan, Italy) and developed at Sigma-Tau (Pomezia, Rome, Italy) and CVie Therapeutics (Hong Kong, China). The pharmacologic characteristics of the compound are described elsewhere (Quadri et al., 1997; Ferrari et al., 1998, 1999a,b).

#### Animal Models

##### Ethical Approval.

Care and husbandry of animals complied with the guidelines of the Prassis Sigma-Tau Research Institute for Animal Care (approved by the Italian Ministry of Health), the European Directive 86/609, and the Italian Law (DLI 16, January 27, 1992). Animals were monitored by a veterinarian.

##### Mouse Model.

The β-adducin–null mouse model (official strain designation 129-Add2tm1Lip/Lip) has been previously described (Gilligan et al., 1999). Wild-type 129/Sv mice, carrying the same β-adducin polymorphism (Q529R) described in the MHS rats (Tripodi et al., 1996), were used as controls. Starting after weaning, male wild-type mice (n = 28) were divided into two groups receiving rostafuroxin (100 μg/kg per day in 0.5% Methocel; Dow Chemical Company, Midland, MI), n = 13) or vehicle (0.5% Methocel, n = 13) by gavage for 25 weeks. Male β-adducin–null mice (n = 10) were used as controls, and two β-adducin–null mice received rostafuroxin (100 μg/kg per day) for 25 weeks.

Mice were used to test the effect of rostafuroxin on the following parameters: 1) systolic blood pressure (SBP) and heart rate (HR), which were recorded weekly in conscious mice by an indirect tail-cuff plethysmographic method (BP recorder; U. Basile, Varese, Italy); 2) urinary parameters, which were measured monthly in conscious mice; and 3) podocyte protein expression. After 25 weeks of rostafuroxin treatment, all mice were sacrificed to prepare renal microsomes for immunoblotting quantifications of podocyte proteins.

##### Rat Models.

Two rat models were studied: 1) the congenic rat substrains from MHS and MNS rats, and 2) the ouabain hypertensive rats.

For the first model, congenic rat substrains from MHS (designated 129-Add2tm1Llp/Llp) has been previously described (Tripodi et al., 1997; Ferrari et al., 1998). Normotensive control rats (MHS) and OHR rats, hypertension was induced in male Sprague-Dawley rats, 5 weeks old and weighing 110–120 g, by subcutaneous infusion of ouabain (15 μg/kg per day, n = 30; Sigma-Aldrich, St. Louis, MO) with osmotic minipumps (Alzet; Charles River, Calco, Italy) for 12 weeks, as previously described (Ferrari et al., 1998). Normotensive control rats (n = 15) received sterile saline solution through osmotic minipumps. After 4 weeks, OHR rats were divided into two groups receiving rostafuroxin (100 μg/kg per day in 0.5% Methocel, n = 15) or vehicle (0.5% Methocel, n = 15) by gavage for 8 weeks.

The effect of rostafuroxin in OHR rats was tested on the following parameters. First, SBP and HR were recorded weekly by an indirect tail-cuff plethysmographic method (BP recorder; U. Basile). The initial SBP of controls and OHR rats was comparable (average 125–130 mm Hg), then it increased in OHR rats as compared with controls. HR was not affected by ouabain treatment. After 12 weeks of ouabain infusion, plasma ouabain levels increased in OHR compared with controls (from 0.2 ± 0.03 to 0.7 ± 0.07 nM; P < 0.001) (Ferrandi et al., 2004). Second, for urinary parameters, at the end of rostafuroxin treatment, rats were acclimatized to metabolic cages, and urine was collected for urine parameters measurement. Finally, for podocyte protein expression, nephrin and synaptopodin were quantified by immunofluorescence and immunoblotting in renal microsomes. The effect of ouabain on nephrin and synaptopodin expression was replicated ex vivo by incubating podocyte primary cell cultures from Sprague-Dawley neonatal rats with 10−9 M ouabain, without or with a simultaneous incubation with 10−9 M rostafuroxin for 5 days.

##### Biochemical Assays for Urinary Parameters in Mice and Rats

Urinary parameters were measured in conscious wild-type and knockout mice monthly, starting from 5 up to 25 months of age. Mice received a 1-ml bolus of physiologic solution by gavage, and urine was collected over a period of 6 hours.

Urinary parameters were also measured in conscious congenic male rats at 3 and 6 months of age and in OHR rats. Rats were housed in individual metabolic cages with free access to water and food. Urine samples were collected over a period of 24 hours and analyzed for the following parameters: volume, pH, osmolality, Na+, and K+ concentrations (IL 943 Electrolyte Analyzer; Instrumentation Laboratory, Milan, Italy), total protein excretion (total protein kit; Sentinel Diagnostics, Milan, Italy), and creatinine (colorimetric kit; Sentinel Diagnostics).


**Immunostaining**

Immunofluorescence was performed on unfixed renal tissue embedded in optimal cutting temperature compound (Miles Scientific, Naperville, IL), the tissue was snap-frozen in a mixture of isopentane and dry ice, and stored at -80°C. Five-micrometer sections were placed on slides and fixed in cold acetone. Cells were seeded on glass coverslips and fixed in buffered paraformaldehyde. In indirect immunofluorescence, the bound primary antibody [anti-neprhin (Progen, Darra, QLD, Australia), anti-synaptopodin (Sigma-Aldrich)] was detected with Alexa Fluor 546 anti-guinea pig or anti-mouse IgG (Invitrogen, Carlsbad, CA). F-Actin was stained with rhodamine-labeled phalloidin (Sigma-Aldrich). Slides were mounted with Vectashield aqueous mounting medium (Vector Laboratories, DBA Italia SRL, Segrate, Italy).

**Renal Microsome Preparations and Caveolae Isolation**

Renal microsomes were prepared from mice and rats. Kidneys were homogenized in 250 mM sucrose, 30 mM histidine, and 1 mM EDTA pH 7.2, and centrifuged at 6000g for 15 minutes. The supernatant was centrifuged at 48,000g for 30 minutes. The pellet, containing renal microsomes, was resuspended in sucrose-histidine buffer and analyzed by Western blotting.

Caveolae-enriched microdomains were purified according to a detergent-free procedure, as previously described (Ferrandi et al., 2004). Briefly, renal tissues were homogenized in 200 mM sodium carbonate (pH 11), 2 mM sodium orthovanadate, 100 mg/ml Pefabloc, and centrifuged at 6000g for 10 minutes. The supernatant was sonicated and fractionated on a 5–45% sucrose gradient. The gradients were centrifuged at 150,000g for 18 hours, and 13 fractions were automatically collected and protein content measured. The expression of the specific proteins in the single fractions was determined by Western blotting. Low-density fractions (fractions 2–5) containing the specific caveolin marker were referred to as caveolae.

**Glomeruli and Podocyte Isolation**

Kidneys were removed from 7- to 10-day-old male and female rats. Glomeruli were isolated by sieving, then seeded in culture flasks precoated with collagen type IV (Sigma-Aldrich) at 37°C in 5% CO₂ atmosphere. On days 4–5, podocyte growth started and, by day 8, glomeruli were detached using trypsin EDTA and filtered through the 36-μm mesh to discard glomeruli. Second-passage podocytes, >90% pure as judged by light microscopy, were seeded in flasks and chamber slides. Cell characterization was performed by immunofluorescence, using markers for podocytes (neprhin, podocin, synaptopodin), epithelial (cytokeratin), a-smooth muscle actin, and endothelial cells (CD31).

Second-passage podocytes from neonatal NA and NB rats were seeded in flasks, incubated for 5 days with 10⁻⁸ M rostafuroxin, and analyzed by Western blotting. Podocytes were also obtained from neonatal Sprague-Dawley rats, incubated with 10⁻⁹ M ouabain alone or in the presence of 10⁻⁹ M rostafuroxin, and analyzed by Western blotting.

Measurement of actin stress fibers in podocytes was performed on digitized images using the ImageJ software (image.nih.gov). After manual selection of the cell area as a region of interest, a color threshold procedure allowed selective highlighting of stress fibers in gray mode. The software was programmed to calculate automatically the area occupied by staining. An operator blind to the experiments evaluated 50 cell images per experimental procedure. Data were exported in an Excel file (Microsoft, Redmond, WA), and mean, S.D., and S.E. were calculated. The statistical differences were then analyzed by analysis of variance.

**Adducin Purification and Tyrosine Kinase Phosphorylation Assay in a Cell-Free System**

Recombinant wild-type and mutant rat α-adducin were prepared according to previously published procedures (Ferrandi et al., 1999).

Tyrosine kinase phosphorylation was measured in a cell-free system by Western blotting. Human recombinant full-length kinases belonging to the Src family (such as Src, Yes, Fyn, Lyn) and Abelos tyrosine-protein kinase 1 (ABL1) and vascular endothelial growth factor receptor 1 (VEGFRFlt-1) (50 ng) (Upstate Millipore, Darmstadt, Germany) were incubated without or with adducin (0.75 μg) in a medium containing 10 mM Tris, 10 mM MgCl₂, 5 mM MnCl₂, 0.25 mM EGTA, 0.025 mM orthovanadate, 80 mM NaCl, and 2 mM ATP, pH 7.4, for 10 minutes at 30°C. Rostafuroxin (concentration range from 10⁻¹³ to 10⁻⁹ M) was simultaneously added to the kinases. The reaction was stopped by adding Laemmli sample buffer, and the samples were then analyzed by Western blotting. Adducin and tyrosine kinase phosphorylation were investigated using an antiphosphotyrosine antibody (clone 4G10; Upstate Millipore) or specific antibodies as indicated.

**Western Blot Analysis**

Samples were prepared by SDS-PAGE (Criterion XT, Bio-Rad, Hercules, CA), blotted on a nitrocellulose membrane (Bio-Rad) for 90 minutes, and incubated overnight at 4°C with specific primary antibodies, followed by 1-hour incubation with fluorescent secondary antibodies [Alexa Fluor, 680 nm, red (Invitrogen); IRDye, 800 nm, green (Rockland, Gilbertsville, PA)]. The Western blotting was analyzed and quantified by the Odyssey Infrared Imaging Detection System (LI-COR Biosciences, Lincoln, NE). The optical densities were expressed as arbitrary units. The following antibodies were used: anti-neprhin (Progen), anti-podocin (Sigma-Aldrich), anti-synaptopodin (Sigma-Aldrich), anti-α-adducin (raised by Dr S. Salardi at Prassis Sigma-Tau), anti–Zona Occludens protein 1 (ZO-1) (Invitrogen), anti-actin (Sigma-Aldrich), anti-phosphotyrosine (clone 4G10; Upstate Millipore), anti-pTyr412 Src (Cell Signaling Technology, Danvers, MA), and anti-pTyr416 ABL1 (Cell Signaling Technology).

**Statistical Analysis**

Data are reported as the mean ± S.E.M. The statistical significance was measured by analysis of variance, and P < 0.05 was considered statistically significant.

**Results**

**β-Adducin–Null Mice**

**Effect of Rostafuroxin on Systolic Blood Pressure.**

SBP did not differ between β-adducin knockout (KO) and age-matched wild-type (WT) mice at any age (Fig. 1A) (Ferrandi et al., 2010b). The chronic oral treatment of WT mice carrying the same β-adducin polymorphism (Q529R) as described in MHS rats (Tripodi et al., 1996) with 100 μg/kg per day rostafuroxin for 25 weeks, starting from weaning, did not affect either SBP (Fig. 1A) or HR (KO 641 ± 5, n = 10; WT 620 ± 12, n = 13; WT + rostafuroxin 628 ± 15 beats/min, n = 13). Analogously, rostafuroxin did not affect SBP or HR in KO mice (KO control, SBP 114 ± 1.4 [Fig. 1A], HR 641 ± 9, n = 10; KO + rostafuroxin, mean SBP 112 mm Hg, mean HR 648 beats/min, n = 2).

**Effect of Rostafuroxin on Renal Function.**

No difference in urinary parameters (volume, Na⁺ and K⁺, creatinine excretion) between KO and WT mice was observed, whereas a significant reduction of urinary protein excretion, either normalized or not for creatinine excretion, was present in KO as compared with WT mice (Fig. 1B) (Ferrandi et al., 2010b). The prolonged oral treatment of WT mice with 100 μg/kg per day rostafuroxin for 25 weeks normalized proteinuria to the level of KO control mice (Fig. 1B). Conversely, rostafuroxin did not affect proteinuria of KO mice (proteinuria/creatininuria:...
KO control, 2.6 ± 0.16, n = 10; Fig. 1B; KO + rostafuroxin, mean 2.58, n = 2).

**Effect of Rostafuroxin on Podocyte Protein Expression.**
A Western blot analysis performed on renal microsomes from KO and WT mice showed that the increased proteinuria in WT mice was accompanied by the reduction of nephrin protein expression, the key component of the podocyte slit diaphragm membrane, compared with KO mice (Fig. 1C) (Ferrandi et al., 2010b). The chronic treatment of WT mice with rostafuroxin restored nephrin protein expression to the level of KO mice (Fig. 1C), thus favoring the re-establishment of a normal podocyte filtration activity. Rostafuroxin did not induce any nephrin modification in KO mice (KO control, 136.4 ± 5.2, n = 10; Fig. 1C; KO + rostafuroxin, mean 136 arbitrary units, n = 2).

**Rat Models**

To further investigate the effects of rostafuroxin, two rat models were studied: 1) the congenic rat substrains from MHS and MNS rats, and 2) the OHR rats.

**Congenic Rat Substrains from MHS and MNS Rats.**

**Effect of rostafuroxin on systolic blood pressure.** Compared with MNS rats, the NA congenic strain showed a significant increase of SBP (Table 1). Conversely, the NB congenic strain showed SBP levels similar to those of MNS rats (Table 1) (Tripodi et al., 2004; Ferrandi et al., 2010b). The prolonged oral treatment with rostafuroxin (100 μg/kg per day for 8 weeks, starting from weaning) reduced SBP in MHS and NA rats, but was inactive in MNS rats (Table 1), confirming previous data (Ferrandi et al., 2010a). HR was significantly lower in MHS as compared with MNS rats, and rostafuroxin treatment did not affect HR in any rat strain (Table 1).

**Effect of rostafuroxin on podocyte protein expression.** MNS rats develop an age-dependent glomerulosclerosis and proteinuria (Ferrandi et al., 2010b), which is sustained by an increased glomerular production of thrombomodulin compared with MHS (Brandis et al., 1986; Pugliese et al., 1986; Salvati et al., 1990). Although the prostanoid alteration is the main cause of this dysfunction, the presence of β-adducin in NB rats contributes to a further deterioration of the glomerular damage with age, as documented by the enhanced proteinuria in MNS rats, which is independent from BP levels (Supplemental Table 1) (Ferrandi et al., 2010b). On the contrary, MHS and NA rats, carrying the mutant α-adducin, appear protected over time (Supplemental Table 1) (Ferrandi et al., 2010b).

Due to the interference exerted by the genetic background of MNS rats, in the present study, we did not investigate the effect of rostafuroxin in vivo in congenic rats since the excessive prostanoid production could mask the effect of the compound on the portion of proteinuria related to the adducin-dependent mechanism.

To overcome this difficulty, and to directly assess the effect of rostafuroxin on podocyte protein expression, glomerular podocytes were isolated and cultured in the presence of the compound. Previous studies have indicated that MNS and NB rats, compared with MHS and NA rats, show an early reduction of the expression of some podocyte proteins, including nephrin, which precedes the development of proteinuria (Ferrandi

### TABLE 1

<table>
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<tr>
<th></th>
<th>Controls</th>
<th>+100 μg/kg per day Rostafuroxin</th>
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<tr>
<td></td>
<td>MHS, n = 8</td>
<td>MNS, n = 8</td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>350 ± 4</td>
<td>375 ± 6</td>
</tr>
<tr>
<td><strong>SBP (mm Hg)</strong></td>
<td>139 ± 1</td>
<td>168 ± 1.2**</td>
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<tr>
<td><strong>HR (beats/min)</strong></td>
<td>398 ± 12</td>
<td>336 ± 1.7**</td>
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**BW:** body weight.

* *SP < 0.01 versus MNS; **SP < 0.01 controls versus rostafuroxin.*
et al., 2010b). Here, we confirmed by Western blot analysis that, in glomerular podocytes from newborn rats, the protein expression of nephrin, podocin, synaptopodin, α-adducin, and ZO-1, but not actin, was significantly downregulated in MNS and NB as compared with NA rats (Fig. 2A). The incubation of cultured podocytes from NB rats with $10^{-9}$ M rostafuroxin for 5 days favored the re-expression of all of these proteins, except for actin, counteracting podocyte protein loss and damage caused by the presence of mutant β-adducin. Conversely, the effect of rostafuroxin was almost absent in NA rat podocytes, since the presence of mutant α-adducin already exerted a protective role from podocyte injury (Fig. 2A).

Furthermore, a fluorescence analysis for phalloidin, a high-affinity probe for filamentous actin, of glomerular podocytes from NB rats showed that mutant β-adducin induced a cytoskeletal damage associated with a shortening of actin filaments, whereas the incubation of podocytes with $10^{-9}$ M rostafuroxin was able to restore actin filament length (+38%, $P < 0.05$) (Fig. 2B).

Collectively, the findings in cultured podocytes suggest that rostafuroxin protects NB rats from podocyte protein loss and cytoskeletal alterations associated with the presence of mutant β-adducin genetic variant.

**Ouabain Hypertensive Rats.** OHR rats were obtained by a chronic ouabain infusion with osmotic minipumps releasing 15 μg/kg per day for 12 weeks, as previously described (Ferrari et al., 1998).

**Effect of rostafuroxin on systolic blood pressure.** Ouabain infusion in rats increased SBP, and rostafuroxin treatment at 100 μg/kg per day for 8 weeks antagonized this ouabain-induced effect (Fig. 3A), confirming previous data (Ferrari et al., 1998). Neither ouabain nor rostafuroxin affected HR (control, 350 ± 6.3, n = 15; OHR, 355 ± 7.5, n = 15; OHR + rostafuroxin, 358 ± 9 beats/min, n = 15).

**Effect of rostafuroxin on renal function.** Ouabain and rostafuroxin did not induce variations of creatinine excretion (control, 31.8 ± 1.16, n = 15; OHR, 30.63 ± 0.95, n = 15; OHR + rostafuroxin, 30.98 ± 1.14 mg/24 hours, n = 15). Conversely, ouabain infusion in rats induced a significant increase of urinary protein excretion (milligrams per 24 hours) (Bignami et al., 2013), which was normalized by the prolonged treatment with rostafuroxin (Fig. 3B). A similar picture was observed when proteinuria was normalized for creatinine excretion (control, 1.19 ± 0.1, n = 15; OHR, 1.95 ± 0.2, n = 15, $P < 0.05$ versus control; OHR + rostafuroxin, 1.2 ± 0.11, n = 15, $P < 0.05$ versus OHR).

**Effect of rostafuroxin on podocyte protein expression.** The significant increase of proteinuria induced by ouabain in OHR
rats was associated with the downregulation of nephrin protein expression (Fig. 3, C and E) but not synaptopodin (Fig. 3, D and E) (Bignami et al., 2013), as measured by Western blot in renal microsomes and immunofluorescence analysis. Prolonged treatment of OHR rats with rostafuroxin restored nephrin protein expression (Fig. 3, C and E), but did not affect synaptopodin (Fig. 3, D and E).

This finding was reproduced ex vivo by incubating primary cultures of normal rat podocytes with 10^{-9} M ouabain, without or with 10^{-9} M rostafuroxin, for 5 days. In this
setting, ouabain lowered nephrin protein expression (Fig. 3F) (Bignami et al., 2013), but did not affect synaptopodin (Fig. 3G), as documented by a Western blot analysis. As observed in vivo, rostafuroxin, simultaneously incubated with ouabain, blunted the effect of ouabain, favoring the re-expression of nephrin (Fig. 3F), without affecting synaptopodin expression (Fig. 3G).

Collectively, the present findings suggest that rostafuroxin counteracts ouabain-induced effects on glomerular podocytes, preventing nephrin protein loss and consequently restoring podocyte function.

**Effect of Rostafuroxin on SH2-Domain Interactions**

We attempted to investigate the molecular mechanism through which rostafuroxin antagonizes mutant β-adducin and ouabain-induced effects on podocytes. We based our hypothesis on previous findings indicating that rostafuroxin antagonizes mutant α-adducin– and ouabain-activated Na,K-ATPase interaction at the Src-SH2 domain, resulting in BP normalization through the modulation of renal tubular Na,K-ATPase activity and its related signaling (Ferrandi et al., 2004, 2010a).

Here, we hypothesized that the compound may recognize other SH2-domain interactions, besides that at Src. This finding may be relevant to address the effect of rostafuroxin in the nephrin-signaling network, since evidence has indicated that SH2-domain interactions are involved in the complex signaling cascade originating from the slit diaphragm (Verma et al., 2006). The tyrosine kinases on which we evaluated the effect of rostafuroxin were selected on the basis of a score matrix–assisted ligand identification analysis performed on nephrin and β-adducin protein sequences. According to this analysis, nephrin may be phosphorylated in tyrosine residues by Fyn, Src, and Yes (Supplemental Fig. 1), as already published (Verma et al., 2003, 2006; Quack et al., 2006), as well as by ABL1, whereas β-adducin appears phosphorylated by Fyn, as known (Gotoh et al., 2006), as well as by Yes (Supplemental Fig. 2).

Interestingly, these identified kinases share a specific sequence for the SH2-binding motif—that is, R-E-FLVRESE in Src, Yes, and ABL1 and R-E-FLIRESE in Fyn and Lyn (Zhao et al., 2013).

Therefore, we set up experimental conditions in a cell-free system to activate the selected tyrosine kinases by using the recombinant wild-type and mutant β-adducin to evaluate whether rostafuroxin may prevent interactions at a selective SH2 domain.

We showed that rostafuroxin inhibited the phosphorylation of ABL1 and Yes when these tyrosine kinases were activated by Src (Supplemental Fig. 3, A and B), with a peak effect at 10⁻¹¹ to 10⁻¹² M, but not by Lyn (Supplemental Fig. 3, C and D). Furthermore, rostafuroxin antagonized the tyrosine phosphorylation of mutant, but not wild-type, β-adducin induced by Yes (Fig. 4A), with a peak effect at 10⁻¹¹ M, but not by Fyn (Fig. 4B). We also showed that rostafuroxin antagonized the activation of Yes (Supplemental Fig. 4A), with a peak effect at 10⁻¹⁰ to 10⁻¹¹ M, but not Fyn (Supplemental Fig. 4B) induced by another substrate, VEGF1/Flt-1.

We started to validate these findings in vivo. In particular, we studied ABL1 membrane localization and its protein expression in congenic rats. We showed that ABL1 is compartmentalized with caveolin1 and nephrin in cavedae microdomains isolated from rat kidneys (Supplemental Fig. 5, A and B). Preliminary data indicated that ABL1 is localized in glomerular podocytes (Supplemental Fig. 5C), and its expression level appears downregulated in MNS and NB congenic rats compared with MHS and NA rats, similar to nephrin (Supplemental Fig. 5D). A positive correlation between nephrin and ABL1 protein expression in glomerular podocytes is shown (Supplemental Fig. 5E).

Collectively, the present findings suggest that rostafuroxin antagonizes selective SH2-domain interactions of tyrosine kinases at the binding motif R-E-FLVRESE, common to Src, ABL1, and Yes, but not R-E-FLIRESE, common to Fyn and Lyn. Although not yet clarified, these results may indicate a possible protective effect of rostafuroxin on podocyte derangement mediated through the modulation of specific SH2-containing proteins/adaptors that may be downstream to nephrin signaling networks.

**Discussion**

The present study uncovers a novel pharmacologic application of rostafuroxin in glomerular diseases mediated by mutant β-adducin and ouabain. By combining mouse and rat models (mice carrying mutant β-adducin, congenic rats for mutant α- and β-adducin derived from MHS rats and ouabain hypertensive rats) and rat podocyte cultures, we produced consistent data indicating that rostafuroxin antagonizes podocyte protein loss and the increased urinary protein excretion induced by mutant β-adducin and ouabain. These effects occur independently from blood pressure modulation.

In particular, studies in mice and NB congenic rats, both carrying the same β-adducin polymorphism (Q529R) described in MHS rats (Tripodi et al., 1996) or in OHR rats, have shown that the development of glomerular podocyte damage and proteinuria is associated with an early downregulation of nephrin. Conversely, conditions that protect from podocyte injury, such as those observed in the presence of mutant α-adducin genetic variant in MHS and NA rats, restore proteinuria and nephrin expression to normal levels. Similar alterations of podocyte protein expression have been reproduced by culturing podocytes from NB congenic rats (Ferrandi et al., 2010b) or by incubating podocytes in the presence of nanomolar concentrations of ouabain (Bignami et al., 2013). These data imply that β-adducin mutation and ouabain induce direct changes in podocyte architecture and activity.

However, the novelty of the present study is the effect of rostafuroxin. For the first time, we show that the in vivo treatment of mice carrying β-adducin mutation and OHR rats with rostafuroxin normalizes podocyte nephrin expression and proteinuria. The effect of rostafuroxin in vivo in NB congenic rats has not been investigated, due to the interference of the genetic background of MNS rats, which favors the development of a form of glomerulosclerosis associated with an excessive glomerular production of prostanoids (Pugliese et al., 1986; Salvati et al., 1990). However, in cultured podocytes, where the effect of tromboxane is removed, rostafuroxin indeed restores podocyte protein expression to normal levels in NB rats, where mutant β-adducin induces a more pronounced damage, more so than in NA rats, which already appear protected. Analogously, rostafuroxin antagonizes ouabain-induced downregulation of
nephrin in ouabain-cultured podocytes as well as in vivo in OHR rats.

The definition of the genetic-molecular mechanisms underlying the different forms of podocyteopathies appears as a useful approach to target treatments and to optimize the response to the pharmacologic therapy. From this study, rostafuroxin has emerged as an important tool to study and identify the genetic-biochemical abnormalities underlying glomerular diseases triggered by adducin mutations and EO/ouabain in genetic and experimental animal models. The compound appears as a novel drug that may also target these altered molecular mechanisms in patients. Indeed, previous findings have indicated that rostafuroxin is a potent and safe drug (Ferrari et al., 1999a,b) able to counteract hypertension development, and the related hypertensive effects, induced by mutant α-adducin and ouabain at renal and vascular levels, preventing cardiac and renal hypertrophy in the long run in animal models (Ferrandi et al., 2004; Zhang et al., 2005). More recently, a pharmacologic clinical trial has shown that rostafuroxin also normalizes BP levels in patients carrying a selective genetic profile that includes genes encoding for adducin variants and enzymes controlling ouabain synthesis and transport (Lanzani et al., 2010).

The rostafuroxin molecular mechanism underlying the antihypertensive effect has been identified in our previous studies. It relies on its ability to inhibit the interaction of mutant α-adducin- or ouabain-activated Na,K-ATPase at the Src-SH2 domain, preventing Src activation and the Src-dependent Na,K-ATPase phosphorylation and activation at the renal tubular level (Ferrandi et al., 2010a). The result is the disruption of the complex signaling cascade downstream to Src-Na,K-ATPase induced by rostafuroxin, which is associated with the normalization of BP in hypertensive rats and patients (Ferrandi et al., 2010a; Lanzani et al., 2010).

Definite protein interaction networks that involve SH2 domain-containing proteins appear to control a wide spectrum of physiologic activities, which include cardiac and renal function, and influence cardiovascular remodeling (Liu et al., 2012; Grahammer et al., 2013). However, podocyte activities and actin cytoskeletal dynamics are known to be integrated in a dynamic signaling hub at the slit diaphragm membrane (Grahammer et al., 2013; New et al., 2013). Alterations in these signaling pathways, frequently caused by genetic mutations within these networks that are responsible for biochemical dysfunctions in proteins and in their related signaling cascades, may progress to glomerular diseases (Wiggina, 2007; Mathieson, 2008). One of the key components of the podocyte signaling hub is nephrin, which appears to be controlled at multiple levels by the modulation of its phosphorylation state via Src family tyrosine kinases (Fyn, Yes, Src) and trafficking (Quack et al., 2006) or by a direct degradation process mediated by calpain (Peltier et al., 2006; Tian et al., 2014).

Interestingly, although we have not yet fully addressed the molecular mechanism through which rostafuroxin prevents nephrin loss at the podocyte level and proteinuria, we have two additional sets of data indicating the following.

First, rostafuroxin appears to recognize and antagonize selective interacting substrates at the SH2-binding motif R-E-FLVERE, common to Src, Yes, and ABL1 kinase, but not at the SH2-binding motif R-E-FLIRESE, common to Fyn and Lyn, where a valine (V) is substituted with an isoleucine (I) (Zhao et al., 2013). Interestingly, it has been documented that mutant, but not wild-type, β-adducin tyrosine phosphorylation induced by Yes is reduced by rostafuroxin as well as Src and Flt-1 activation of Yes. Since nephrin is also under the control of Yes, it remains to be established whether rostafuroxin may affect Yes-mediated nephrin interaction and phosphorylation with potential consequences on its functional activity.

**Fig. 4.** Effect of rostafuroxin on in vitro phosphorylation of tyrosine kinases containing SH2 domains. Recombinant β-adducin (0.75 µg) was incubated in a cell-free system for 10 minutes at 30°C with tyrosine kinases (50 ng). A tyrosine kinase containing the SH2-binding motif R-E-FLVERE (Yes) and one containing the SH2-binding motif R-E-FLIRESE (Fyn) were chosen. The effect of increasing concentrations of rostafuroxin (range from 10⁻¹² to 10⁻⁶ M), simultaneously added to the kinases, was investigated. The reaction was stopped by adding Laemmli sample buffer, and samples were analyzed by Western blotting for adducin phosphorylation using an anti-phospho-tyrosine antibody. (A) Effect of rostafuroxin (rost) on mutant (left) and wild-type (right) β-adducin (Add) tyrosine phosphorylation induced by Yes. (B) Effect of rostafuroxin on mutant (left) and wild-type (right) β-adducin tyrosine phosphorylation induced by Fyn. Data are the mean ± S.E.M. of four independent experiments. *P < 0.05 +rostafuroxin versus –rostafuroxin (black bar). pTyr, phospho-tyrosine.
We started to investigate one of the selected kinases, ABL1, in in vivo studies. In rat kidneys, preliminary results indicate that ABL1 localizes with nephrin in caveolae subdomains, known to functionally organize signaling pathways (Simons et al., 2001), is present in rat glomerular podocytes, and its expression level is downregulated in MNS and NB glomerular podocytes, as well as nephrin, compared with MHS and NA.

Although the relevance of this finding in the context of the nephrin-signaling network still remains to be elucidated, ABL1 kinase appears of particular interest since a score-matrix-assisted ligand identification analysis suggests that nephrin contains three possible SH2-consensus sequences for ABL1 (Supplemental Fig. 1), not yet functionally identified, that, if verified, may indicate the involvement of this kinase in the modulation of nephrin signaling function.

Indeed, nephrin-associated kinases are implicated in the recruitment of SH2-SH3 domain-containing adapter proteins and in the phosphorylation of substrate proteins belonging to the podocyte junctions, favoring the assembly of actin filaments and the integrity of the podocyte signaling network (Verma et al., 2006).

Although verification is still needed, the results of the present study may imply that mutant β-adducin and EO/ouabain affect signaling pathways downstream to nephrin, which include interactions with SH2-containing substrate proteins, or adaptors, responsible for podocyte derangements.

Rostafuroxin may exert a selective activity on specific SH2-containing domains preventing the sequence of events leading to deleterious podocyte lesions.

Second, mutant β-adducin and ouabain increase calpain activity in cultured podocytes, and this effect associates with nephrin cleavage (M. Ferrandi, personal communication). This hypothesis is further reinforced by the evidence that, in mesenteric arteries of OHR rats, ouabain increases the activity of α2 Na,K-ATPase, Na-Ca exchanger 1, and TRPC6 channels in a Src-dependent manner, being responsible for the increased intracellular Ca$^{2+}$ (Zulian et al., 2013). Since rostafuroxin blocks adducin- and ouabain-induced effects, we can envisage that the compound may also affect Ca$^{2+}$ signaling in glomerular podocytes, thus preventing mutant β-adducin- and ouabain-mediated nephrin cleavage induced by calpain or, possibly, the TRPC6-PLC-γ1-mediated pathway for nephrin signal transduction regulation.

Certainly, the identification of a successful treatment for glomerular diseases is still a great medical need, and new therapeutic strategies are highly desirable. Clinical evidence supports the notion that, once the glomerular disease is established, it may progress to terminal renal failure (Levey and Coresh, 2012). Therefore, in clinical practice, it is crucial to find a drug which can block the initial triggering events but also delay, or prevent, the progression to end-stage renal disease. Although not completely understood, the molecular mechanisms that operate in chronic renal failure causing glomerular damage, loss of active nephrons, proliferation of mesangial cells, and thickening of basal lamina are frequently associated with tubule-interstitial fibrosis, renal tissue inflammation, and matrix deposition that may ultimately induce a compensatory hypertrophy of the remaining nephrons and of the whole kidney (Turner et al., 2012). Peptides, such as angiotensin II, or the increased hydrostatic pressure, responsible for mechanical stress, and the rearrangement of actin cytoskeleton mediated by Rho family GTPases and reactive oxygen species (Babelova et al., 2013) have been considered the most likely cause of podocyte injury. From the present study, mutant β-adducin and increased levels of EO/ouabain emerge as additional causes of podocytopathies.

Interestingly, rostafuroxin counteracts adducin-mediated cytoskeletal alterations and ouabain prohypertrophic effects (Ferrandi et al., 2004) and prevents proteinuria occurrence in animal models. Therefore, additional studies should be undertaken to establish whether the observations in animal models may be translated to patients, thus indicating if rostafuroxin may be a drug of choice to antagonize mutant β-adducin- and ouabain-mediated effects in human glomerular diseases. This observation, if verified, becomes of particular relevance since it opens a novel therapeutic option for patients who experience glomerular disease progression associated with these two mechanisms.

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References


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