Nebivolol Attenuates Maladaptive Proximal Tubule Remodeling in Transgenic Rats

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Key Words

NADPH oxidase • Proximal tubule cell • Megalin

Abstract

Background/Aims: The impact of nebivolol therapy on the renal proximal tubular cell (PTC) structure and function was investigated in a transgenic (TG) rodent model of hypertension and the cardiometabolic syndrome. The TG Ren2 rat develops nephropathy with proteinuria, increased renal angiotensin II levels and oxidative stress, and PTC remodeling. Nebivolol, a β_1 -antagonist, has recently been shown to reduce albuminuria, in part, through reductions in renal oxidative stress. Accordingly, we hypothesized that nebivolol therapy would attenuate PTC damage and tubulointerstitial fibrosis. **Methods:** Young Ren2 (R2-N) and SD (SD-N) rats were treated with nebivolol (10 mg/kg/day) or vehicle (R2-C; SD-C) for 3 weeks. PTC structure and function were tested using transmission electron microscopy and functional measurements. **Results:** Nebivolol treatment decreased urinary N-acetyl- β -D-glucosaminidase, tubulointerstitial ultrastructural remodeling and fibrosis, NADPH oxidase activity, 3-nitrotyrosine levels, and increased megalin and lysosomal-associated membrane protein-2 immunostaining in PTCs. Ul-

trastructural abnormalities that were improved with therapy included altered canalicular structure, reduced endosomes/lysosomes and PTC vacuoles, basement membrane thickening, and mitochondrial remodeling/fragmentation. **Conclusion:** These observations support the notion that nebivolol may improve PTC reabsorption of albumin and other glomerular filtered small molecular weight proteins in association with the attenuation of oxidative stress, tubulointerstitial injury and fibrosis in this rat model of metabolic kidney disease.

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Introduction

Increases in the prevalence of the metabolic syndrome and diabetes play an important role in the increasing incidence of chronic kidney disease [1–3]. The histopathological feature that is most strongly linked to progressive renal impairment is proximal tubule cell (PTC) injury and tubulointerstitial fibrosis [4–6]. Microalbuminuria is an important early clinical indicator of progressive kidney disease in association with the metabolic syndrome and diabetes [1–3]. PTCs normally reabsorb most of the

glomerular-filtered protein, so only 30 mg or less appears in the urine [7–14]. Further, there is increasing evidence that growth factors such as angiotensin II (Ang II), in conjunction with filtered proteins, can exert direct injury to the PTC resulting in tubulointerstitial fibrosis.

Albumin and other small molecular proteins filtered through the glomerulus are processed by PTC by at least two distinct pathways and most of the filtered albumin is returned to the peritubular blood by a retrieval pathway [11–15]. Compelling evidence for the importance of this retrieval pathway has been provided by two-photon microscopy [16]. These studies have demonstrated that large vesicles laden with intact albumin track through the PTC from the apical to the basolateral regions. This process is disrupted by elevated tissue levels of Ang II. Albumin retrieval can be restored through Ang II blockade [7, 9–20]. Smaller quantities of filtered proteins that are not retrieved undergo lysosomal degradation before urinary excretion as small peptide fragments [17, 21]. This degradation pathway is especially susceptible to metabolic and growth factors such as Ang II, responsible for maladaptive changes in the PTC and consequent tubulointerstitial fibrosis [10–20].

A continuous and extensive microvilli brush border covers the apical portion of the PTC providing the increased surface area responsible for receptor-mediated endocytosis of albumin and other small molecular weight proteins. The protein megalin is localized in the brush border microvilli in association with canalicular structures, clathrin-coated pits, endocytic vesicles and recycling endosomes, and facilitates this receptor-mediated protein endocytosis [21, 22]. Albumin and other small molecular weight proteins are subsequently disassociated from these receptors, degraded into their constituent polypeptides and amino acids via the endosomal/lysosomal degradation pathway, and then transported across the basement membrane of the PTC and absorbed by adjacent interstitial capillaries [22, 23].

There is evidence that activation of the kidney renin-angiotensin system (RAS) plays a crucial role in PTC maladaptive structural remodeling and functional abnormalities by increasing oxidative stress [24–29]. Increased intrarenal Ang II has been reported in transgenic Ren2 rats [23–26, 30], which also manifest activation of the sympathetic nervous system (SNS) as reflected by elevated levels of norepinephrine [31]. In this regard, β -adrenergic receptor blockers suppress the SNS tissue response and renal secretion of renin [32, 33]. These data facilitate the notion that β -blockers could reduce PTC injury and remodeling in scenarios involving both RAS

and SNS activation such as exists in the transgenic Ren2 rat that manifests progressive albuminuria [3, 7, 26].

Nebivolol is a β_1 -antagonist blocker which is known to increase tissue nitric oxide (NO) bioavailability and reduce NADPH oxidase activity [34–38]. Currently, there are limited studies exploring the impact of nebivolol treatment on the kidney in animal models displaying tubulointerstitial injury [39–42]. Recently, we observed that nebivolol treatment in Ren2 rats reduced proteinuria, and increased the podocyte-specific markers podocin and desmin [34]. Interestingly, the glomerular protective effects of nebivolol were very modest compared with prior intervention studies utilizing RAS blockade [26, 28] and could not explain the substantive reductions in albuminuria that were observed [34]. To further explore its renal protective effects, we investigated the impact of nebivolol treatment on PTC and tubulointerstitial abnormalities in Ren2 rats.

Material and Methods

Animals and Treatments

Male Ren2 and age-matched SD rats (6–9 weeks) were randomly assigned to control (R2-C and SD-C) or nebivolol-treated groups (R2-N and SD-N) ($n = 5$). Nebivolol-treated rats received 10 mg/kg/day released via an implanted osmotic mini-pump for 21 days. All procedures were approved by the University of Missouri Animal Care Committees and housed in accordance with NIH guidelines. Blood pressure was measured in triplicate using the tail-cuff method prior to initiation of treatment and prior to sacrifice at 21 days [34]. Urine protein and β -NAG were determined using automated colorimetric assays [9, 34].

Transmission Electron Microscopy (TEM)

Renal cortical tissue was thinly sliced and placed immediately in primary TEM fixative stained with 5% uranyl acetate and triple-lead stain [9, 26]. To maintain uniformity, only S-1 segments of the PTC with identifiable microvilli that were immediately adjacent to glomeruli were examined.

Megalín- and Lysosomal-Associated Membrane Protein-2 Immunohistochemistry

Briefly, 4- μ m sections were incubated with 1:50 goat anti-megalin or megalín- and lysosomal-associated membrane protein-2 (LAMP2) antibody overnight and then incubated with 1:300 anti-goat Alexa flour 647 for 4 h and viewed with a confocal laser-scanning microscope. Images were captured by LSM imaging system and intensities quantified by MetaVue as average gray scale intensities.

NADPH Oxidase and Subunits (Rac1 and p47^{phox}) and 3-Nitrotyrosine (3-NT) Content

NADPH oxidase activity was determined in kidney cortical tissue by a spectrophotometric technique [9, 26]. Rac1 and p47^{phox} sections were incubated with 1:200 Rac1 primary antibody or

1:100 goat polyclonal p47^{phox} in tenfold diluted blocking agent overnight. Sections were incubated with 1:300 anti-goat for p47^{phox} and anti-mouse for Rac1 for 4 h; signal intensities were then analyzed [26]. For 3-NT, sections were incubated with 1:200 primary rabbit polyclonal anti-nitrotyrosine antibody overnight, washed and incubated with secondary antibodies, biotinylated linked and streptavidin HRP for 30 min each. Diaminobenzidine was applied for 7 min, sections rinsed again and stained with hematoxylin for 45 s, rehydrated, and images captured and signal intensities quantified [26].

α -Smooth Muscle Actin (α -SMA) and Collagen III Co-Staining: Fibrosis

Dual staining was used to quantify the level of collagen type III in the adventitia and α -SMA in the media of the arteries in the proximal tubules areas. Briefly, the sections were incubated first with 1:50 rabbit anti-collagen type III and then with 1:50 mouse anti- α -SMA overnight, washed and stained with 1:300 mixed donkey and anti-rabbit (collagen type II) and donkey anti-mouse (α -SMA) and signal intensities quantified.

VVG Staining for Tubulointerstitial Fibrosis

Sections were evaluated with VVG stain as previously described [28]. Slides were viewed with a Nikon 50i microscope and images captured.

Statistical Analysis

All values are expressed as mean $\pm$ SE. Statistical analyses were performed in SPSS 13.0 using ANOVA with Fisher's LSD and Student's t test for paired analysis.

Results

Nebivolol Reduced Systolic Blood Pressure, Urinary Protein and β -NAG

Administration of nebivolol resulted in a small systolic blood pressure reduction and urinary protein excretion in the Ren2 [34]. In the R2-N there was a trend in the R2-C for increased urinary β -NAG that was reduced in the R2-N ($p < 0.05$) (fig. 1a).

Nebivolol Attenuates Abnormal Ultrastructural Remodeling in the PTC

PTC of R2-C decreased the number and length of the electron-dense canaliculi, decreased dense apical tubules, and endosomes (fig. 1b). Nebivolol increased in both number and length of canaliculi so that they appeared similar to the SD-C.

Nebivolol Treatment Restored Decreased Megalin in the Ren2 Tubulointerstitium

Megalin immunostaining was substantially reduced in the tubulointerstitium of R2-C ($p < 0.05$) and normalized following nebivolol treatment for 21 days (fig. 2). In-

creases in megalin paralleled reductions in both albumin and β -NAG with nebivolol treatment. Since megalin has been implicated in lysosomal biogenesis [15], this could help explain the decreased number of lysosomes in R2 kidneys and their restoration with 3 weeks of nebivolol treatment (fig. 3).

Nebivolol Increases the Number of Lysosomes and Transcytotic Vesicles-Vacuoles

There were decreased numbers of electron-dense lysosomes and electron-lucent transcytotic vesicles-vacuoles in the R2-C (fig. 3a), and treatment increased the number of both lysosomes and transcytotic vesicles-vacuoles (R2-N). In concert with the decreased lysosome numbers, LAMP2 was reduced in the Ren-C and restored following nebivolol treatment (fig. 3b).

Nebivolol Attenuates Tubulointerstitial Oxidative Stress in Ren2

NADPH oxidase activity was increased in the R2-C tubulointerstitium and restored to similar levels of the SD-C following nebivolol treatment ($p < 0.05$) (fig. 4a). A similar pattern was observed with subunits Rac1 and p47^{phox} (fig 4b). A marker for peroxynitrite formation, 3-NT, was increased in the R2-C and decreased following treatment with nebivolol ($p < 0.05$) (fig. 4c).

Nebivolol Attenuates Early Perivascular and Tubulointerstitial Fibrosis

R2-C displayed marked basement membrane thickening in PTCs (fig. 5) attenuated with nebivolol treatment. Remodeling of the tubulointerstitium consisted of both periarterial adventitial expansion of extracellular matrix and tubulointerstitial fibrosis (fig. 6) findings attenuated with nebivolol treatment ($p < 0.05$). The R2-C tubulointerstitial regions also demonstrated spherical enlargement, loss of elongation of mitochondria and the ultrastructural changes were improved with treatment (not shown).

Discussion

Our understanding of the pathophysiology of PTC injury in response to Ang II and/or other growth factors and metabolic insults is very limited. However, the tubulointerstitial structural and functional abnormalities in the Ren2 kidney in this study are consistent with previously observed Ang II and associated oxidative stress-mediated injury [9, 26]. Treatment with nebivolol for 3

Fig. 1. Nebivolol improves urinary (β -NAG) and PTC endosomal remodeling in the Ren2 (R2). **a** β -NAG is reduced with nebivolol treatment in the R2 [$** p < 0.05$ treatment (+) compared to control (-)]. **b** Representative TEM images for ultrastructural analysis of the PTC endosomal region. R2 had fewer clathrin-coated pits (inset) at the base of the microvillar brush border and decreased number and length of the electron-dense, elongated canalicular structures (arrows) at the base of the microvilli (bottom left). Nebivolol treatment in the R2 (bottom right) restores both length and number of the electron-dense elongated canalicular structures in the endosomal region of the PTC. Mv = Microvilli.

weeks substantially reduced tubulointerstitial oxidative stress and fibrosis as well as PTC structural abnormalities. In addition to reducing urinary albumin [34], nebivolol treatment substantially reduced urinary β -NAG. This small molecular weight protein is a 140-kDa lysosomal enzyme that is present in high concentrations in PTC but is not typically excreted into the urine by normal PTCs. Increased β -NAG is a marker for altered function and/or injury to the PTC [9, 43]. Thus, in addition to reducing

NADPH oxidase activity, 3-NT levels, fibrosis and PTC ultrastructural structural abnormalities, this treatment also corrected the PTC functional abnormality as assessed by urine β -NAG levels [9, 43].

The decrease in urine β -NAG levels were paralleled by increased PTC megalin immunostaining in Ren2 following nebivolol treatment. Megalin is an endocytic receptor, belonging to the low-density lipoprotein family, which is well expressed in the PTC brush border, luminal

Fig. 2. Nebivolol restores megalin in the Ren2 (R2). Representative images from immunohistochemistry analysis of megalin (a) with corresponding average gray scale intensities (b). ** $p < 0.05$ treatment (+) in R2 compared to control (-).

endosomes and lysosomes [8, 21, 22]. Its importance is evidenced by the fact that megalin-deficient mice have increased amounts of albumin and several other low molecular weight proteins in the urine [21, 22]. Consistent with our TEM observations in Ren2 PTC, reduced megalin has been associated with loss of PTC endocytic invaginations/vesicles, reduced lysosomes and loss of canicular integrity [21, 22]. Extant data suggests that megalin may be involved in albumin reabsorption directly as a receptor albumin, and indirectly by affecting the ex-

pression of cubilin which is coexpressed with megalin in the PTC brush border and the endocytic apparatus [21, 22]. Albumin is subsequently disassociated from these PTC receptors (megalin and cubilin) and transported via the endosomal/lysosomal degradation pathway, where it is degraded into its constituent polypeptides and amino acids. These degraded constituents are then transported across the basement membrane of the PTC and absorbed by adjacent interstitial capillaries and returned to the circulation [22, 23]. This endocytic pathway is especially susceptible to metabolic and growth factors such as Ang II. In this regard, a recent study has demonstrated that increased Ang type 1A receptor-mediated growth pathway signaling reduces megalin expression in cultured PTCs [29]. Our data would suggest that this Ang II-mediated reduction in megalin expression can be corrected with an antihypertensive agent which reduces renal oxidant stress.

Data from this study suggests that nebivolol may correct the structural and functional PTC abnormalities seen in Ren2 transgenic rats, in part by reducing NADPH oxidase and associated increases in tubulointerstitial oxidative stress. In this regard, treatment with nebivolol, like AT₁R blockade [9], attenuates PTC injury in this model characterized by increased renal Ang II levels [9, 26]. Ang II generates superoxide anions (O_2^-) in cardiovascular tissue and the kidney largely by increasing NADPH oxidase activity that potentiates inflammation and fibrosis [44, 45]. Despite our observation that nebivolol treatment improved mitochondrial structure, it is likely that the reduction in oxidative stress primarily reflected a reduction in NADPH oxidase activity rather than reductions in mitochondrial O_2^- production [45–49]. These data collectively suggest that an increase in NADPH oxidase is a convergent pathway by which increased Ang II and/or albuminuria causes PTC injury, and that antihypertensive agents that reduce renal NADPH oxidase [9, 35–39] protect against tubulointerstitial fibrosis and preserve PTC ability to process albumin and other small molecular proteins that are filtered through the glomerulus.

The results of this investigation support the notion that a vasodilating β -blocker, unlike conventional β -blockers, provides renal-protective actions. A clinical study in hypertensive patients with type 2 diabetes showed that a vasodilating β -blocker, carvedilol, but not metoprolol, reduced albuminuria on top of RAS blockade [50]. The authors concluded that the beneficial effect of carvedilol may be related to an ability to reduce oxidative stress. It is suggested that nebivolol, like carvedilol, may

Fig. 3. Nebivolol restores decreased PTC lysosomes, apical and basilar vacuoles, and LAMP2 in the Ren2 (R2). **a** Representative TEM images of ultrastructural analysis of the R2 S-1 region of the PTC. R2-C (middle image) demonstrate decreased intense electron-dense (black) ovoid lysosomes (arrows) and electron-lucent (white) apical and basilar vacuoles (white arrows) compared to SD-C (left image) restored with nebivolol treatment in the R2-N (right

image). Below are higher magnification images to better demonstrate the electron-lucent basilar vacuoles. Cap = Capillary. **b** Representative images from immunohistochemistry analysis of LAMP2 with corresponding average gray scale intensities (**c**). * p < 0.05 untreated (-) R2 controls (-) compared to untreated (-) SD controls; ** p < 0.05 treatment (+) compared to control (-).

Fig. 4. Nebivolol attenuates kidney cortical tissue NADPH oxidase, subunits (Rac1 and p47^{phox}), and 3-NT content in the transgenic Ren2 (R2) model. **a** Total NADPH oxidase enzyme activity. **b** Representative images of immunohistochemistry analysis of the NADPH oxidase subunits Rac1 and p47^{phox} subunits with corresponding average gray scale intensities below. **c** 3-NT staining as a marker of peroxynitrite (ONOO⁻) formation. * $p < 0.05$ untreated (-) R2 control (-) compared to untreated (-) SD control; ** $p < 0.05$ treatment (+) compared to untreated control (-).

Fig. 5. Nebivolol attenuates PTC basement membrane (BM) thickening in the Ren2 (R2). Top panel represents images of the normal PTC BM in SD-C with no demonstrable remodeling changes following nebivolol treatment (SD-N). Bottom panel depicts the two types of BM thickening: the cytosolic layering-addition thickening (X) in the R2-C (top left). The more prevalent electron-dense finger-like protrusion BM thickening (bottom left) depicts the extracellular matrix thickening following the path of the invaginating basilar canaliculi. Note: nebivolol attenuates this BM thickening in the R2 (right images – R2-N). Mt = Mitochondria.

Fig. 6. Nebivolol attenuates perivascular arteriolar and tubulointerstitial fibrosis in the Ren2 (R2) model. **a** Representative images of immunohistochemistry analysis of α -smooth muscle actin (green; colors in the online version only) and collagen type III (red) in interstitial arterioles with gray scale intensities below (left) and average area of periarteriolar fibrosis (right). **b** Repre-

sentative VVG staining of tubulointerstitial regions with corresponding measure of intensity (left) and average area (right). * $p < 0.05$ untreated (-) R2 control (-) compared to untreated (-) SD-C; ** $p < 0.05$ treatment (+) compared to untreated control (-).

also have renal-protective effects because of the beneficial effects of this drug to improve bioavailable endothelial derived NO and reduce oxidative stress [51]. Our results within vivo nebivolol treatment in transgenic rats are commensurate with this notion. In this regard, we have observed both reduced glomerular [34–39] and tubulointerstitial NADPH oxidase activity and 3-NT levels, consistent with prior observations in cardiovascular tissue

[36–39]. Similarly, our observation that nebivolol treatment reduced both peritubular and perivascular fibrosis is consistent with a previous experimental study comparing nebivolol and atenolol in a renal mass reduction [41] and those in a rat model of contrast-induced nephropathy [42] which demonstrated that nebivolol, but not atenolol, reduced renal fibrosis and improved NO-endothelial function. The authors concluded that reduced renal fi-

bro sis was attributable to decreased oxidative stress and/or increased NO bioavailability in response to nebivolol but not atenolol treatment. Current data are consistent with the notion that nebivolol reduces tubulointerstitial oxidative stress along with fibrosis and other indicators of PTC functional and structural abnormalities.

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