Protective actions of nebivolol on chronic nitric oxide synthase inhibition-induced hypertension and chronic kidney disease in the rat: a comparison with angiotensin II receptor blockade

Natasha C. Moningka1, Tatsiana Tsarova1, Jennifer M. Sasser1 and Chris Baylis1,2

1Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL, USA and 2Department of Medicine, University of Florida, Gainesville, FL, USA

Correspondence and offprint requests to: Natasha C. Moningka; E-mail: nmoningk@ufl.edu

Abstract

Background. Nitric oxide (NO) deficiency contributes to chronic kidney disease (CKD) progression and hypertension. The β-blocker, nebivolol (N), also enhances NO production, and we studied whether N attenuates CKD and hypertension caused by chronic NO synthase inhibition (CNOSI).

Methods. Male Sprague–Dawley rats on 6 weeks of CNOSI (L-NAME, 150 mg/L drinking water) received placebo (P), N (10 mg/kg/day), olmesartan (O, 2.5 mg/kg/day) or N + O. Blood pressure (BP) and urine protein and NOx (metabolites of NO) were monitored throughout. We measured glomerular sclerosis (GS), creatinine clearance (CCr) and components of the NO and oxidant pathways in the renal cortex.

Results. BP increased >50 mmHg in P by weeks 4–6, but no change occurred in N, O or N + O. P rats developed proteinuria and GS and CCr was ~30% of normal. In N, O and N + O all values remained normal. In renal cortex of P, p22phox and nitrotyrosine abundance as well as H2O2 levels were higher and extracellular superoxide dismutase (EC SOD) was lower versus normal kidneys. N, O and N + O normalized p22phox, H2O2 and EC SOD and increased Mn SOD above normal. The cortical neuronal NO synthase (nNOS) β abundance increased in P and this was prevented by N, O and N + O.

Conclusion. We suggest that the major benefit from both N and O is reduction in oxidative stress in the renal cortex, which may potentiate residual local NO. There was no additive benefit of N + O since each drug effectively prevented injury, but a combination may be beneficial where protection is incomplete with each drug. The increased nNOSβ protein seen early in the course of the CKD may contribute to the evolving GS.

Keywords: creatinine clearance; glomerular sclerosis; L-NAME; olmesartan; superoxide dismutase

Introduction

Reduction of nitric oxide (NO) production and/or availability is associated with endothelial dysfunction, hypertension and the associated cardiovascular risk and progression of chronic kidney disease (CKD) [1]. In rats, chronic NO synthase inhibition (CNOSI) leads to hypertension and CKD which can be prevented by inhibition of the renin–angiotensin system (RAS) with converting enzyme inhibitors and with angiotensin II (ANGII) type 1 (AT1) receptor inhibition [2]. This suggests that CNOSI activates the RAS and that a change in the balance between NO and ANGII is responsible for the hypertension and end organ damage associated with NO deficiency. Indeed, RAS inhibition is the first line treatment for control of progression of CKD [3] and is also widely used for treatment of high blood pressure (BP) [4].

An alternative or additional therapeutic strategy would be an intervention to restore NO production, and the β-adrenergic antagonist nebivolol enhances NO production/activity by several mechanisms [5]. In some parts of the vasculature and in the heart, nebivolol directly stimulates endothelial NO synthase (eNOS) protein abundance and activity, and some of this effect is via β-3 adrenoceptor activation [5–11]. In vitro, nebivolol increases degradation of the NOS inhibitor, asymmetric dimethylarginine (ADMA), by activating ADMA’s catalyzing enzyme, dimethylarginine dimethylaminohydrolase (DDAH), at the message, protein and enzyme activity level [12]. Nebivolol also prevents accumulation of ADMA in vivo [13–15].

Nebivolol exerts antioxidant actions including inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [5, 16–19], which preserves NO bioavailability by preventing NO uncoupling and peroxynitrite formation. Additional protective effects of nebivolol include the classical β-1 adrenergic blockade-induced antihypertensive actions, both via direct effects on the heart and indirect reduction of renin release [20].

Fortepiani et al. [21] reported that nebivolol attenuates the CNOSI-induced hypertension produced by 14 days of
chronic L-NAME administration to the rat. In the present study, we experimentally induced CNOSI with high-dose L-NAME for 6 weeks to produce systemic hypertension and CKD [2, 22] in order to investigate whether nebivolol influenced the pathways that control NO availability. Since chronic RAS inhibition is protective in this model [2] and is used clinically for control of CKD progression, we compared the actions of nebivolol to those of olmesartan, an angiotensin type 1 (AT1) receptor antagonist. Furthermore, we investigated whether a combination of nebivolol and AT1 receptor antagonism might have additive protective effects.

Materials and methods

Animals

All aspects of rat handling were approved and monitored by the University of Florida Institutional Animal Care and Use Committee. Studies were performed in 39 male Sprague–Dawley (SD) rats (from Dublin, VA facility; Harlan Laboratories, Indianapolis, IN) at 10–12 weeks of age. Rats were allowed ad libitum drinking water and rat chow and during acclimatization were introduced to daily aliquots of half teaspoon chocolate pudding (vehicle for nebivolol delivery; ConAgra Foods, Inc, Omaha, Nebraska); intake was monitored. Rats reluctant to eat the pudding were excluded from further study. Some rats were subjected to surgery for telemetric measurement of BP and heart rate (HR) and allowed 7–10 days recovery.

On days for metabolic cage measurements, rats were placed on a low nitrate, complete diet (AIN-76C, MP Biomedical, Solon, OH) and were then placed in a metabolic cage for 16 h with access to distilled water but without food. All rats were then given 1.5 mg/100 g body weight (BW) L-NAME (Sigma, St Louis, MO) IV (via tail vein), followed by 150 mg/L L-NAME in the drinking water (−15 mg/kg/24 h) in 0.1% NaHCO3 and 5% ethanol (vehicle for olmesartan) throughout the study and randomized as follows: placebo (P), rats received two daily aliquots of half teaspoon of chocolate pudding (n = 9); nebivolol (N; Forest Research Institute, Jersey City, NJ) rats received 2 × daily aliquots of half teaspoon of chocolate pudding each containing 5 mg/kg of nebivolol (to deliver a total daily dose of 10 mg/kg/day) (n = 8); olmesartan (O; LGM Pharma, Inc, Boca Raton, FL), in which rats received olmesartan to deliver ~2.5 mg/kg/day (25 mg/L in drinking water) and 2 × daily aliquots of half teaspoon chocolate pudding (n = 7); and nebivolol + olmesartan (N + O), in which rats received 25 mg/L olmesartan in water and 2 × half a teaspoon of chocolate pudding each containing 5 mg/kg of nebivolol (n = 10). Drinking and pudding consumption were monitored daily to ensure that rats were ingesting the correct drug doses.

BW was measured at baseline and then 2 to 3 × weekly and 16 h metabolic cage measurements were performed at Weeks 2, 4, 5 and 6 of study for overnight urine collection. On these days, the nebivolol was given as a single dose (10 mg/kg/day) −8 h before rats were placed in metabolic cages. Urine samples were stored at −80°C for later analysis. BP and HR were measured by telemetry in 5/9 P rats, in 7/8 N rats, in 5/7 O rats and 7/10 N + O rats over a 24-h period at baseline and at 2-week intervals. After the final measurements, rats were anesthetized with isoflurane, a needle was inserted into the abdominal aorta and a terminal blood sample taken, centrifuged and plasma stored at −80°C. The vasculature was perfused with cold phosphate-buffered saline, the left kidney removed and weighed and a mid-coronal section was cut and placed in 10% buffered formalin. The right kidney was also removed and both the remaining part of the left kidney and the right kidney were separated into cortex and medulla, flash frozen in liquid N2 and stored at −80°C.

Preparation for telemetry

In a preliminary operation, under isoflurane anesthesia and using a full sterile technique, a catheter was fed under the skin by trocar and introduced into the femoral artery of 24 rats. The catheter was tied into position, and the Caro transmitter unit was sutured to the internal abdominal wall. After recovery, rats were singly housed, and BP/HR were measured using the Data Sciences International (DSI) equipment and software (St Paul, MN).

An additional five age-matched SD rats were used as an untreated control group. After a 16 h metabolic cage urine collection, rats were sacrificed and tissues were harvested under the same conditions as above.

Chemical analyses

Plasma and urine creatinine were measured by high-performance liquid chromatography [23] and plasma, urine and tissue NOx by Griess assay [24]. Urine protein was measured by the Bradford assay (Bio-Rad, Hercules, CA). Kidney cortex levels of hydrogen peroxide (H2O2) normalized to total protein (nmol/mg) were measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes) according to the manufacturer’s instructions. Specificity of the assay was confirmed by incubation with catalase (2000 U) [25].

Western blot

Individual protein abundance was measured by western blot on 200 µg kidney cortex protein as follows: for eNOS, neuronal nitro synthase (nNOS) α and β and nitrotyrosine, samples were loaded on 7.5% gels, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (25–35 mA, 2.5 h) and blotted onto nitrocellulose membranes (1 h, 0.46 Amps; Amersham Biosciences, Piscataway, NJ). Membranes were blocked and probed with either the eNOS antibody (1:250; BD Transduction, San Jose, CA), the C-terminal rabbit polyclonal antibody to the nNOS (1:500, overnight incubation; Thermo Fisher Scientific, Waltham, MA) for the nNOSβ, the N-terminal rabbit polyclonal nNOS antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) for nNOSα and the mouse monoclonal nitrotyrosine antibody (1:500; Millipore, Temecula, CA). Secondary antibodies were used goat anti-mouse (170-6516; Bio-Rad) at 1:2000 for eNOS and nitrotyrosine and 1:3000 for nNOSβ or goat anti-rabbit (170-6515; Bio-Rad) at 1:3000 for nNOSα.

All other proteins were loaded onto 12% gels separated at 140 V for 65 min. For superoxide dismutase (SOD) isoforms, we used Stressgen (Ann Arbor, MI) antibodies; #SOD-101 for CuZn SOD, 1:2000; #SOD-111 for Mn SOD at 1:2000 and Abcam (ab 21974; Cambridge, England) at 1:250 for extracellular superoxide dismutase (EC SOD). Secondary antibodies were used goat anti-rabbit (Bio-Rad 170-6515) at 1:2000. For p22phox and dimethyldiaminohydrolase (DDAH) isoforms 1 and 2, we used Santa Cruz antibodies: p22phox, sc-11712, 1:50 dilution; DDAH1, sc-26068, 1:250; DDAH2, sc-32859, 1:250. Secondary antibodies were used donkey anti-goat (sc-2020) at 1:2000. For protein methyltransferase-1 (PRMT1), we used Millipore (070-404; Billerica, MA) at 1:2000 and goat anti-rabbit at 1:2000 for the secondary. Bands were visualized using ECL (Thermo Fisher Scientific) and quantified by densitometry using the VersaDoc (Bio-Rad). Protein abundance was calculated as integrated optical density (minus background) factored for Poncpeau Red stain (Sigma) and then normalized to control or placebo.

Histology

Fixed kidney was blocked in paraffin wax and 5 µm thick sections were cut and stained with periodic acid with schiff with hematoxylin/eosin counterstain (Sigma). Sections were examined, blind, for the level of glomerular sclerosis (GS) and tubulointerstitial injury. Up to one hundred glomeruli were scored, blinded, as follows: 0, healthy glomeruli; +1, <25% damage; +2, 25–50% damage; +3, 51–74% damage; +4, >75% damage. A GS index score was calculated using the following equation: (6(# of +1) + 2(# of +2) + 3(# of +3) + 4(# of +4))/total glomeruli observed.

Statistics

Data are presented as mean ± SE. Statistical analyses were by unpaired Student’s t-test for comparing C and P, one-way analysis of variance with Bonferroni correction for four group comparisons, and by Kruskal–Wallis one-way analysis for pathology. Significance was defined as P < 0.05.

Results

In the rats receiving CNOSI alone (P), only three of nine completed the 6-week observation period; three were sacrificed at Week 4 and three at Week 5 due to rapid deterioration. All rats in the treated CNOSI groups (given N, O and N + O) survived until the 6th week. The BW was similar at baseline (Week 0) and increased up to Week 4 in all three treatment groups. Serial BP was measured by telemetry in some rats in each group and
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mean BP at baseline (Week 0) was normal and similar in all rats (113 ± 1 mmHg) (Figure 2). BP increased with CNOSI in P and was significantly higher than baseline at Week 4 (P < 0.001); only one of the five rats instrumented with telemetry probes in this group was alive at Week 6 of the study. There was no change in BP over the 6-week period in any of the three treatment groups (Figure 2). In P, the HR was not different at 4–6 weeks compared to baseline (386 ± 26 versus 373 ± 7 b.p.m.), whereas HR was reduced at 6 weeks of N (294 ± 3 versus 368 ± 4 b.p.m., P < 0.001), N + O (301 ± 4 versus 346 ± 6 b.p.m., P < 0.001) and O (311 ± 4 versus 354 ± 4 b.p.m., P < 0.005).

The 24 h urinary excretion of NOx (UNOxV) was similar in all groups at baseline and fell significantly at Weeks 4 and 6 in P (both P < 0.01), at Weeks 2, 4 and 6 in O (all P < 0.001), at Weeks 4 and 6 in N and N + O (both P < 0.05), Figure 3. At baseline, the urine protein excretion (UprotV) was low and similar in all groups and was elevated by Week 6 in P (P < 0.05) and similar to baseline in the three treatment groups (Figure 4). The 24 h creatinine clearance (CGC) measured just before sacrifice was lower in P compared to the three treatment groups (Figure 5). There were many more casts in cortex and medulla in P (30 ± 4/section) than in N (7 ± 3), O (9 ± 4) and N + O (5 ± 3); all P < 0.05 versus P.

To investigate the impact of CNOSI and treatments on renal NOS and antioxidant/antioxidants in the kidney cortex, we first compared the impact of CNOSI alone (P group) with normal control (C) age-matched rats. As shown in Figure 6, CNOSI had no impact on either eNOS or nNOSα protein abundance, while nNOSβ protein was markedly increased compared to C. There was increased abundance of PRMT1 with CNOSI, a fall in DDAH1 and no change in DDAH2 compared to C (Figure 7). The EC SOD abundance fell (no change in the other SODs; Figure 8A), whereas p22phox, nitrotyrosine (Figure 8B) and kidney cortex levels of H2O2 (Figure 8C) rose with CNOSI versus C.

We then compared the four CNOSI groups and as shown in Figure 9, there was no effect of N, O or N + O treatment versus P on either eNOS or nNOSα protein abundance compared to P. There was no change in kidney cortex PRMT1 abundance with any of the drugs. Nebivolol alone and in combination with O increased the DDAH1 abundance in kidney cortex versus P, while O alone increased DDAH2 abundance compared to P (Figure 10). All three treatments increased EC SOD and Mn SOD (Figure 11A) and reduced p22phox (Figure 11B) compared to P. Nitrotyrosine protein abundance also showed similar
trends; however, only \( N + O \) reached statistical significance (Figure 11B); \( N \) versus \( P \) (\( P = 0.06 \)) and \( O \) versus \( P \) (\( P = 0.08 \)). Kidney cortex levels of \( \text{H}_2\text{O}_2 \) effectively decreased in all treatment groups (Figure 11C).

**Discussion**

The main novel findings from this study are that nebivolol is highly effective in preventing the hypertension, falls in kidney function and structural damage that result from
6 weeks of CNOSI. CNOSI produced marked reductions in total NO production, based on falls in 24 h UNOxV in the untreated, P group, which were also seen with N, O and N + O. Nebivolol had no impact on eNOS or nNOS abundance in the kidney cortex. There were reductions in p22phox, nitrotyrosine and hydrogen peroxide with nebivolol (suggesting a reduction in NADPH dependent superoxide production and therefore oxidative stress) as well as enhancement of both EC SOD and Mn SOD isoforms, which collectively would combat the oxidative stress in kidney cortex. In fact, nebivolol was as effective as the AT1 receptor inhibitor, olmesartan, in affording functional and structural protection in this model. Since each drug effectively eliminated the injury, there was no additive therapeutic benefit when the two drugs were combined.

In this study, we confirm that CNOSI with high-dose L-NAME produces significant systemic hypertension as well as proteinuria and kidney damage in the SD rat. The SD rats used here developed greater hypertension and falls in 24 h Ccr, and exhibited increased mortality compared to an earlier series reported by us [18]. This difference may be because we now obtain our rats from the Harlan facility in Dublin, VA rather than from Harlan Indianapolis, IN. Since this change, we have also noticed an increased susceptibility to CKD/hypertension due to severe renal mass reduction (Y. L. Tain, G. F. Chen, and C. Baylis, unpublished observations). It is noteworthy that SD rats from different vendors also exhibit different susceptibility to CNOSI [26].

In the present study, nebivolol protected against CNOSI-induced hypertension and kidney injury. Nebivolol and olmesartan individually and in combination with nebivolol also afforded substantial antihypertensive and kidney sparing actions, although the fall in UNOxV was not prevented. While this may seem counterintuitive, the magnitude of reduction in total NO production with CNOSI does not necessarily correlate with indices of functional and structural damage. The Wistar Furth (WF) rat is resistant to hypertension and kidney damage with CNOSI compared to the SD, despite marked reductions in UNOxV to similar levels seen in SD [22]. Since NO acts locally to regulate vascular tone and kidney function/structure, small regional changes in NO availability may have important functional effects but are lost when total NO production is estimated. We, therefore, focused on the kidney and found that CNOSI did not produce any differences in the abundance of the two major NOS proteins in the kidney cortex, eNOS.

Fig. 8. Antioxidant and oxidative stress assessment in normal control (C) and in placebo-treated rats receiving 4–6 weeks of chronic L-NAME (P). (A) The protein abundance (factored for total protein loaded determined by ponceau red) in kidney cortex of the antioxidant SODs; EC SOD, copper zinc/cytosolic (CuZn SOD) and manganese/mitochondrial (Mn SOD) SOD isoforms. (B) The protein abundance (factored for total protein loaded determined by ponceau red) in the kidney cortex of the p22phox subunit of NADPHase oxidase and nitrotyrosine. (C) Kidney cortex levels of hydrogen peroxide content. See supplementary figures for representative blots. * denotes P < 0.05 versus P.
or nNOSα compared to controls. Furthermore, neither nebivolol nor olmesartan treatment affected these proteins. In contrast, the nNOSβ isoform, which is present in only small quantities in the healthy kidney, is markedly activated by CNOSI in P, while all three treatments reduced nNOSβ abundance (the implications of this in the context of kidney injury are discussed below). Thus, the only NOS isoform to be affected was increased with injury and reduced with treatment. These findings were unanticipated, however, there are considerable post-translational modifications of NOS activity, which could lead to changes in local NO availability which do not track with protein abundance. One major post-translational modifier of NOS activity is the level of oxidative stress, and there were striking pro-oxidant changes seen in the untreated CNOSI (P) versus C, with loss of EC SOD, increased p22phox (a critical NADPH oxidase subunit), increased nitrotyrosine (a marker of protein nitration likely due to increased peroxynitrite formation) and increased kidney cortex H2O2. All three treatments restored EC SOD and returned p22phox to normal and also elevated Mn SOD above the value of the placebo group. Increases in kidney cortex nitrotyrosine and H2O2 levels were also ameliorated by drug treatment. Nebivolol has been reported to have significant antioxidant actions [16–18], and the increased NAPDH-dependent superoxide production in the kidney of the Ren2 hypertensive rat is attenuated by nebivolol treatment [19]. The present study is the first to report that nebivolol restores EC SOD and enhances Mn SOD abundance in the kidney, which will, together with reduction in p22phox and thence NADPH oxidase activity, provide powerful antioxidant protection. Of note, the antioxidant effects of nebivolol in this study are similar to those of the AT1 receptor blocker olmesartan.

While glomerular hypertension and GS does develop in this model [27], when systemic hypertension is severe and afferent arteriolar resistance very high, there is more glomerular ischemia and small vessel injury [2]. The sclerotic injury to the glomeruli in the present study was quite mild, but the 24 h C Cr was reduced by 66% in the untreated CNOSI rats, suggesting an intense renal vasoconstriction. There was no fall in the abundance of the kidney cortex nNOSα with CNOSI, consistent with only mild glomerular structural injury. In other CKD models, when glomerular damage is severe, we see falls in the kidney cortex nNOSα abundance, which may be related to the CKD progression [1]. We did observe marked increases in nNOSβ abundance in P, an effect also seen with different models of kidney injury [28, 29]. We had previously suggested that the rise in nNOSβ abundance was an attempt at compensation for loss of nNOSα [28], but this is clearly not the case in the present study with the CNOSI model. In fact, we now suggest that the increased kidney cortex nNOSβ abundance contributes to the injury process. Our findings that nebivolol prevent the increased kidney cortex nNOSβ

![Fig. 9.](image1.png)

![Fig. 10.](image2.png)
abundance at the same time as preventing the kidney damage support this notion.

This model is created by pharmacologic levels of the NOS inhibitor L-NAME, thus any changes in the endogenous NOS inhibitor, ADMA, would have no impact on the disease progression. However, we observed a large increase in the abundance of PRMT1 with CNOSI, a fall in DDAH1, and no change in DDAH2 abundance compared to C (Figure 7). These changes would occur in the presence of increased oxidative stress [30]. Surprisingly, none of the treatment groups significantly lowered PRMT1 compared to P, despite an improvement in the antioxidant status. However, nebivolol alone and in combination increased kidney cortex DDAH1 abundance versus P, while O alone increased DDAH2 abundance (Figure 10). These changes may be due to the improved antioxidant status due to treatment [30].

It should be noted that since nebivolol exerts powerful antihypertensive actions in the CNOSI rat model of CKD, this BP lowering effect could contribute importantly to the renal protection. However, a previous study by Ikeda et al. [31] has shown that while both hydralazine and spironolactone exert equivalent antihypertensive actions during NOS inhibition with L-NAME, only spironolactone is renal protective, preserving renal function and structure and preventing proteinuria and renal inflammation. Thus, prevention of the CNOSI-induced hypertension alone is not sufficient to prevent the kidney damage. Furthermore, nebivolol treatment following 5/6 renal ablation/infarction reduces the albuminuria (compared to untreated rats or those receiving metoprolol) but was without antihypertensive effects [32]. Finally, nebivolol had similar antihypertensive actions to atenolol in the slowly evolving 5/6 nephrectomy CKD model but only nebivolol prevented the renal structural injury and oxidative stress [33]. Overall, this suggests that the intrarenal actions of nebivolol, rather than its antihypertensive actions play a major role in the renoprotective effects of the drug.

In summary, nebivolol is equally effective as AT1 receptor blockade in preventing the hypertension falls in glomerular filtration rate and renal structural damage in the CNOSI model. The response of the kidney cortex in terms of NOS, SOD and p22phox protein abundance to these two different drugs was strikingly similar. There was no additive benefit when N + O were combined because each drug individually exerted near complete protection, but in patients with CKD, combination may provide added benefit.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org
Conflict of interest statement. This study was supported, in part, by a contract from Forest Research Institute.

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Received for publication: 25.2.11; Accepted in revised form: 30.6.11