

Salt-Induced Renal Injury in Spontaneously Hypertensive Rats: Effects of Nebivolol

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Key Words

Salt · Hypertension · Kidney · Oxidative stress · Nitric oxide · β_1 -Receptor antagonism

Abstract

Background: We investigated renal effects of nebivolol, a selective β_1 -receptor blocker with additional antioxidative ability, in spontaneously hypertensive rats (SHR) where increased salt intake induces oxidative stress and worsens renal function as a result of further activation of the renin-angiotensin and sympathetic nervous systems. **Methods:** Male SHR were given an 8% salt diet (HS; n = 22) for 5 weeks; their age-matched controls (n = 9) received standard chow. Nebivolol was given at a dose of 10 mg/kg/day for 5 weeks in 11 HS rats. **Results:** HS increased blood pressure, plasma renin concentration, urinary protein excretion, and renal nitrooxidative stress while decreasing renal blood flow and angiotensin 1–7 receptor (mas) protein expression. There was no change in angiotensin II type 1 receptor expression among the experimental groups. Nebivolol did not alter the salt-induced increase in blood pressure but reduced urinary protein excretion, plasma renin concentration, and nitrooxidative stress. Nebivolol also increased neuronal NOS expression while preventing the salt-induced decrease in renal blood flow and mas protein expression. **Conclusion:** Nebivo-

lol prevented salt-induced kidney injury and associated proteinuria in SHR through a blood pressure-independent mechanism. Its protective effects may be related to reduction in oxidative stress, increases in neuronal NOS and restoration of angiotensin II type 1/mas receptor balance.

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Introduction

Recent studies from our group and other laboratories suggest that salt excess is an important determinant of cardiovascular and renal derangement in hypertension [1–3]. Besides its hemodynamic effects, dietary salt excess exerts additional non-pressure-related tissue maladaptive effects in different forms of experimental and human essential hypertension. Increased sympathetic (SNS) [4] and renin-angiotensin system (RAS) activity [5–9], along with increased production of superoxide and other reactive oxygen species [10–13], are among the mechanisms suggested to be involved in the salt-induced hypertension, proteinuria, and progression of early renal injury to nephrosclerosis. In this regard, renal NADPH oxidase activity has been implicated in superoxide production [14–16] and salt-loading increases expression of its subunits, gp91 phox and p47 phox [11, 17].

On the other hand, nitric oxide (NO) exerts a protective role against renal damage in several animal models of kidney disease [18–22] as well as in human chronic renal failure [23, 24]. NO promotes sodium excretion and increases renal blood flow (RBF) [25] and exerts anti-growth and antiproliferative effects on vascular smooth muscle and mesangial cells [26], all of which may counterbalance, at least in part, the detrimental effects of increased SNS and RAS in rats exposed to a high salt diet. Three NO synthase (NOS) isoforms, endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) NOS mediate NO production; however, their regulation in response to excessive salt intake is still controversial. Thus, depending on the renal region and animal model studied, an increased [27, 28], no change [27–29] or diminished [29–32] renal expression of various NOS isoforms in response to salt loading was reported. Importantly, there is no information on expression of NOS isoforms during progression of renal disease in spontaneously hypertensive rats (SHR) fed high salt diet, a recognized experimental model of human essential hypertension. Since superoxide scavenges NO, its reduced availability may also contribute to the development of salt-induced renal injury and dysfunction.

Nebivolol is a highly selective β_1 -receptor blocker with additional direct antioxidant and vasodilator/anti-proliferative potential related to an increased NO bioavailability [33, 34]. Nebivolol reduced blood pressure and decreased NADPH oxidase activity as a main source of superoxide production in the hypertensive kidney of mRen2 transgenic rats while preventing the reduction in eNOS protein expression [15, 16]. In that way, nebivolol corrected NO/superoxide imbalance resulting in diminished proteinuria. The effects of nebivolol on the other NOS isoforms, nNOS and iNOS, have not been investigated, and its renal effects under the conditions of high salt intake in the hypertensives have yet to be determined. Therefore, it is of interest to examine whether nebivolol improves renal dysfunction and remodeling in salt-loaded SHR in view of its potential to not only decrease hemodynamic burden but also to correct renal tissue NO/superoxide imbalance due to high salt intake. Since β -antagonists suppress not only SNS but also RAS, and angiotensin II (Ang II) and angiotensin-(1–7) [Ang-(1–7)] acting upon their receptors [Ang II type 1 (AT₁) and mas receptor, respectively] exert opposing actions on oxidative stress and renal hemodynamics and injury [35–38], we also examined the effects of nebivolol on renal receptor protein expression of these counterbalancing peptides.

Methods

Animals

Male SHR (Charles River), weighing 180–200 g were randomly divided into two groups to receive either a control (1% NaCl; n = 9) or a high salt (8% NaCl; n = 22) diet for the ensuing 5 weeks. The salt-loaded SHR were randomized to one of two groups: vehicle (n = 11) and nebivolol-treated (n = 11) SHR. Nebivolol was given once a day (10 mg/kg) in suspension of 5% gum arabica by gastric gavage. All rats were permitted free access to chow and tap water and were maintained in a temperature and humidity-controlled room with a 12-hour light/dark cycle. Three untreated rats on high salt diet died before the end of the study period. None of the control or nebivolol-treated rats died during the course of the experiment. Systolic blood pressure was measured by tail-cuff plethysmography (Narco Bio System, Houston, Tex., USA) during a baseline period followed by weekly measurements in rats trained beforehand. After 5 weeks on the respective treatment, animals were placed in the metabolic cages for 24-hour urine collections and assessment of protein and creatinine excretion. After urine collection, the animals were decapitated, and trunk blood was collected for measurements of plasma renin concentration (PRC), serum nitrate and nitrite concentrations (NO_x), and serum creatinine. Kidney cortex was collected for analysis of NOS, gp91phox, AT₁, and mas protein expression, NO_x as well as fibrosis and immunostaining for nitrotyrosine. Additional groups of rats kept under the same experimental conditions (n = 5–6 rats/group) were subjected at the end of the experiments to tracheal intubation and right carotid artery cannulation with a transducer-tipped catheter (Micro-Tip 2F, Millar Instruments) for direct measurement of mean arterial pressure (MAP) under pentobarbital anesthesia (50 mg/kg) using a digital data acquisition system (EMKA Technologies, Falls Church, Va., USA). RBF was measured in the same animals as described previously [39] using Transonic flow probe connected to a flowmeter (Transonic System Inc., Ithaca, N.Y., USA) after careful dissection of renal artery and 1-hour adjustment period with saline infusion via PE tubing in the right femoral vein at a rate of 0.4 ml/100 g⁻¹/h⁻¹. The rats were handled in accordance with National Institute of Health guidelines; our Institutional Animal Care and Use Committee approved the study in advance.

Biochemical Assays

Urinary protein was measured by a Bradford protein assay (Bio-Rad, Hercules, Calif., USA). Serum and urinary creatinine were measured with a Quantichrom Creatinine Assay Kit (Bioassay Systems, Hayward, Calif., USA). Creatinine clearance as a measure of glomerular filtration rate (GFR) was calculated from its urinary excretion rate divided by serum concentration, and the data were expressed as milliliters per minute. Blood was collected in EDTA-containing tubes for the assay of PRC. PRC was defined as the rate of Ang I generation from renin in the sample incubated at pH 6.5 for 90 min with excess exogenous substrate provided from nephrectomized rat plasma. Ang I generated in the sample was quantified by radioimmunoassay (Diosarin Corp, Stillwater, Minn., USA). NO_x in serum and tissue samples was determined using a Griess assay (nitrate/nitrite colorimetric assay kit, Alexis Biomedicals, Calif., USA).

Western Blotting

As described previously in detail [40], kidneys were removed and cortical tissue samples were snap frozen in liquid nitrogen and stored at -80°C . Frozen tissue was homogenized in a buffer containing 10 mM HEPES (pH 7.4), 125 mM NaCl, 1 mM EDTA, 1 mM NaF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, and 1 mM PMSF final concentrations. Homogenates were centrifuged at 2,000 and then at 100,000 g for 60 min at 4°C . Protein concentration in both fractions (pellet and supernatant), was determined by Bradford method using BioRad kit (Bio-Rad). Samples of pellets for eNOS, gp91phox, AT_1 , and mas and supernatant for nNOS and iNOS analysis were separated by gel electrophoresis, and then proteins were eluted from the gels to Hybond PVDF membranes (Bio-Rad) for 1 h at 100 V, except for NOS which was transferred for 3 h at 80 V. Nonspecific binding was blocked in 5% nonfat dried milk in 0.1% Tween 20 in TBS for 60 min at room temperature except for the AT_1 receptor which was blocked by Starting Block™ Blocking Buffer (Thermo Scientific, Rockford, Ill., USA). The blots were incubated with a monoclonal anti- β -actin (1:2,000; Sigma, St. Louis, Mo., USA), monoclonal anti- α -tubulin (1:1,000, Abcam, Cambridge, Mass., USA), monoclonal anti-eNOS (1:500; Transduction Laboratories, Lexington, Ky., USA), monoclonal anti-nNOS (1:2,000; BD Transduction Laboratories), monoclonal anti-iNOS (1:2,000; Transduction Laboratories), monoclonal anti-gp91phox (1:1,000; BD Transduction Laboratories), polyclonal anti- AT_1 (1:250; Alomone Labs, Israel), and polyclonal anti-mas (1:200; Alomone Labs). The blots were then incubated with either the secondary anti-rabbit antibody (1:5,000; Amersham Biosciences, Piscataway, N.J., USA) or anti-mouse antibodies (1:3,000 except for α -tubulin for which 1:1,000 was used; Amersham Biosciences). Immunoblots were then resolved with Pierce Super Signal West Pico Chemiluminescent substrates as described by the manufacturer, and exposed to Amersham Hyperfilm-enhanced chemiluminescence (Amersham Biosciences). As positive controls, human endothelial cell lysate for eNOS, rat cerebrum cell lysate for nNOS, mouse macrophage IFN/LPS lysate for iNOS were used. Signal quantification was performed using an image analysis program (MCID), and optical densities were expressed as the ratio between corresponding protein and β -actin or α -tubulin for membrane and cytosolic fraction, respectively. The data were reported as the percentage of the controls.

Histological Analysis

Fixed paraffin sections of kidneys were evaluated for 3-nitrotyrosine (3-NT) immunostaining as a marker for nitroxidative stress as described previously [16]. Fibrosis was evaluated on slides stained with Verhoeff-van Gieson, which is specific for fibrosis and stains elastin (black), nuclei (blue black), collagen (red), and connective tissue (yellow) [41]. Slides were evaluated under a bright-field microscope (model 50i, Nikon), and $\times 40$ images (for 3-NT) or $\times 10$ (for fibrosis) were captured with a Cool Snap camera. Images were analyzed, and signal intensities were measured with MetaVue software (Boyce Scientific, Gary Summit, Mo., USA).

Statistics

All values are expressed as the mean \pm 1 SEM. Data were analyzed by use of ANOVA followed by Newman-Keuls' post-test. A value of $p < 0.05$ was considered to be of statistical significance.

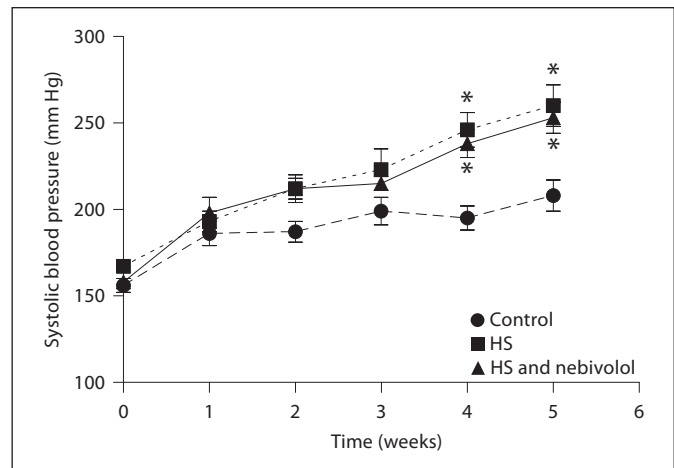


Fig. 1. Effects of high salt diet and nebivolol on time course of systolic tail-cuff blood pressure in SHR. * $p < 0.05$ vs. control.

Results

As already showed in our previous studies, tail-cuff blood pressure progressively increased in untreated salt-loaded SHR when compared to the rats fed control diet. Importantly, nebivolol failed to prevent or to ameliorate the salt-induced increase in blood pressure (fig. 1). Direct measures of MAP at the completion of the treatment regimen averaged 164 ± 7 mm Hg in control, 200 ± 11 mm Hg in untreated salt-loaded rats, and 195 ± 7 mm Hg in salt-loaded rats treated with nebivolol; these data confirmed the absence of an antihypertensive effect of nebivolol.

Renal studies revealed that high salt intake for 5 weeks induced extensive urinary protein excretion in SHR associated with decreased RBF and creatinine clearance (fig. 2). While nebivolol treatment significantly ameliorated excessive urinary protein loss and increased RBF, it failed to improve creatinine clearance.

Serum NOx levels were measured as an index of NO production in the whole body as opposed to tissue NOx levels which evaluated only the local kidney cortex production. As shown in figure 3, there was no difference in serum NOx between the three experimental groups. In contrast, kidney cortex NOx was increased in untreated SHR fed the high salt diet and nebivolol prevented the salt-induced increase in NO production in the kidneys. Consistent with local NOx production, high salt diet increased eNOS protein expression while nebivolol reduced it to levels not different from those found in the control SHR. In contrast, high salt diet did not affect the nNOS

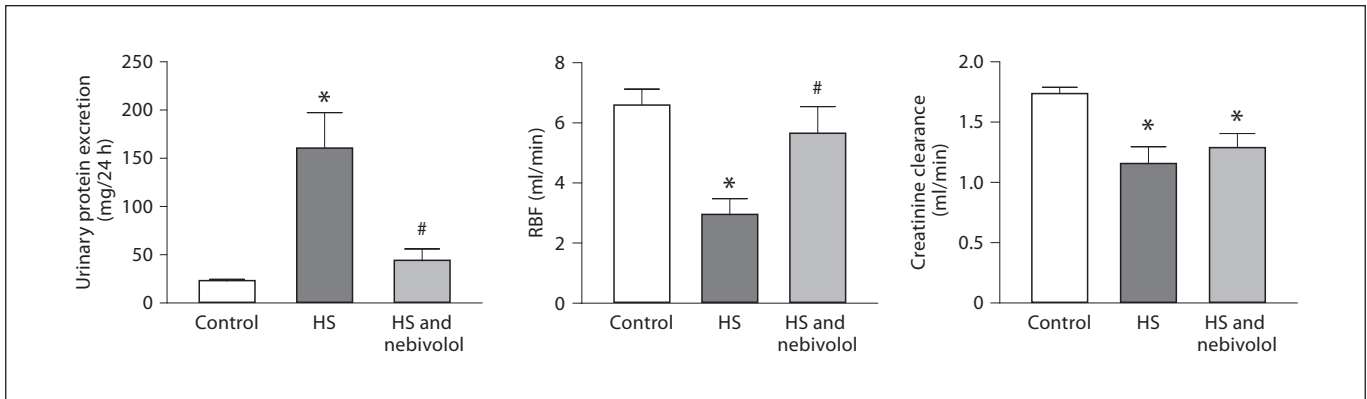


Fig. 2. Effects of high salt diet and nebulolol treatment on renal function. * $p < 0.05$ vs. control; # $p < 0.05$ vs. untreated salt-loaded group.

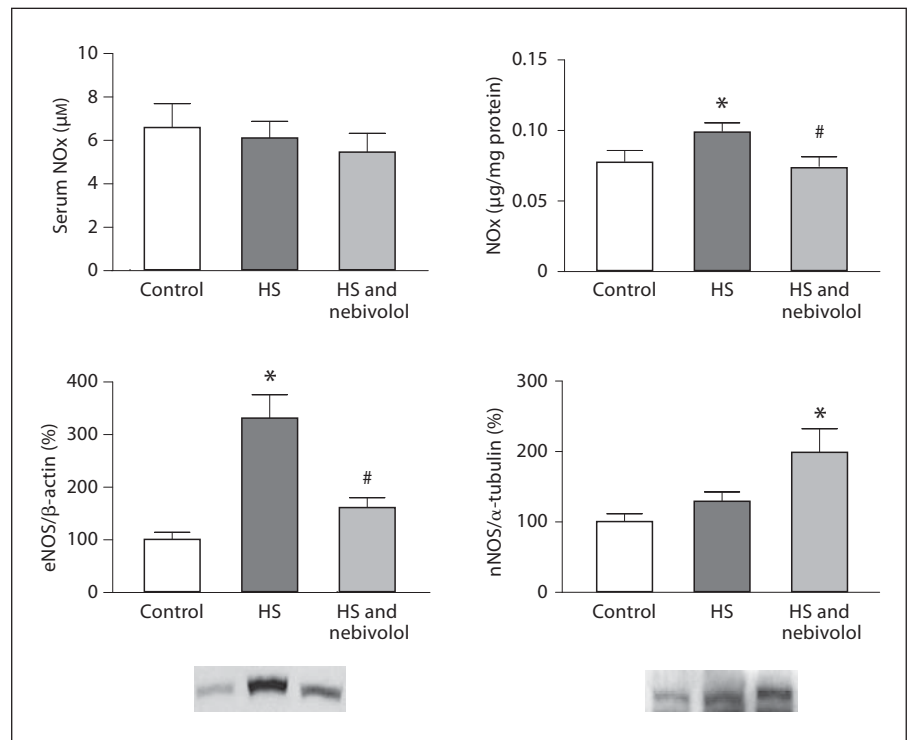
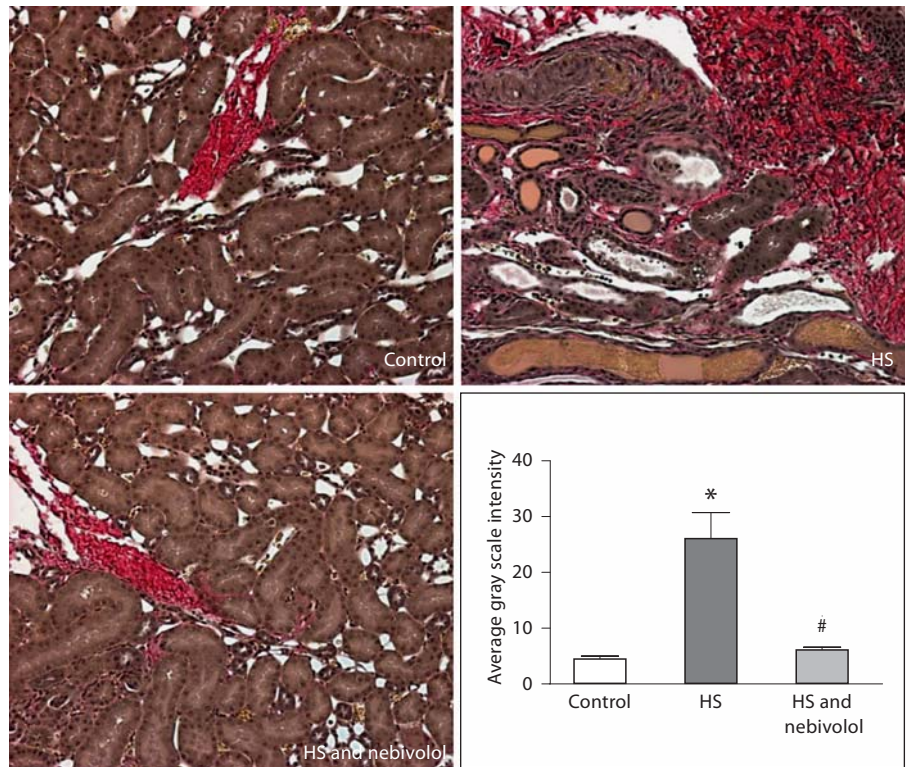
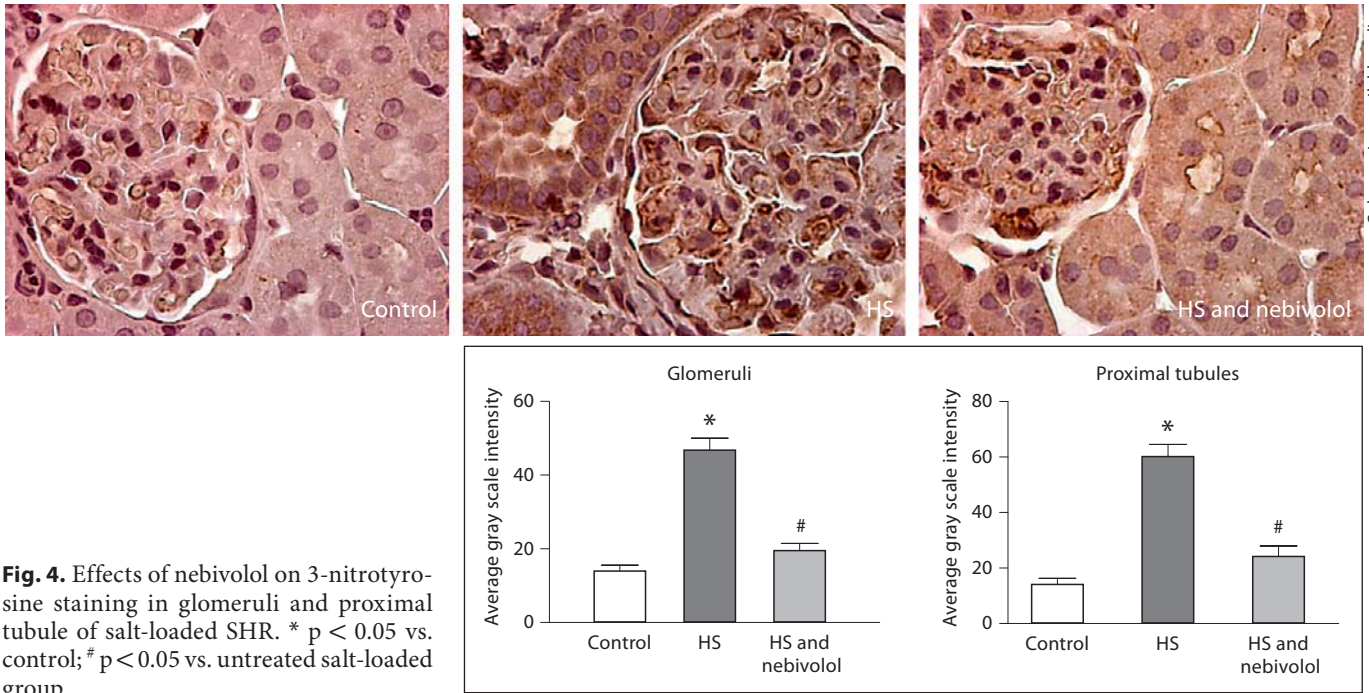


Fig. 3. Serum and renal cortical NO_x, eNOS and nNOS in the control and untreated and nebulolol-treated SHR fed high salt diet. * $p < 0.05$ vs. control; # $p < 0.05$ vs. untreated salt-loaded group.

expression, but nebulolol treatment increased it when compared to the control group. We did not detect iNOS isoform in any of the three experimental groups (data not shown).

The protein expression of gp91phox component of NADPH oxidase was enhanced in renal cortical tissue of untreated SHR fed a high salt diet when compared to the

controls ($224 \pm 27\%$, $p < 0.05$); nebulolol treatment reduced gp91phox protein expression to levels not different from the control animals ($139 \pm 30\%$, $p > 0.05$). Consistent with parallel changes in renal gp91phox protein expression and NO_x production, we demonstrated an increased 3-NT immunostaining and fibrosis in salt-loaded SHR, and nebulolol reduced these changes (fig. 4, 5).



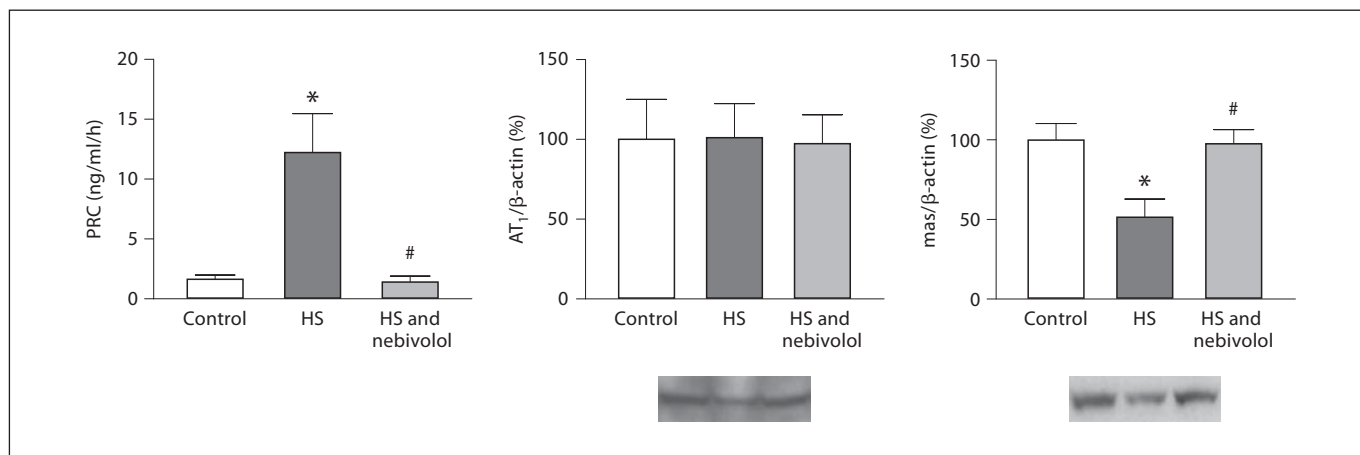


Fig. 6. PRC and renal AT₁ and mas receptor protein expression in control, salt-loaded, and nebivolol-treated SHR. AT₁ = AT₁ receptor; mas = mas receptor. * *p* < 0.05 vs. control; # *p* < 0.05 vs. untreated salt-loaded group.

We confirmed our previous findings that salt loading in SHR increased PRC [9]. We now extended these studies including analysis of renal protein expression for AT₁ and mas receptor. Salt loading decreased mas protein expression, whereas nebivolol prevented this decrease while suppressing salt-induced increase in PRC (fig. 6). There were no changes in AT₁ receptor expression between the three experimental groups.

Discussion

Our results demonstrated that salt-induced renal dysfunction and remodeling in SHR are associated with enhanced nitroxidative stress reflecting an increase in kidney protein expression of eNOS and gp91phox component of NADPH oxidase. In agreement with these results, increased PRC in untreated salt-loaded rats indicates activation of the circulatory Ang II [9], whereas reduced mas protein expression suggests suppressed Ang-(1-7) signaling. Nebivolol treatment of high salt diet-fed SHR did not prevent the salt-induced increase in blood pressure; however, it improved RBF and prevented excessive urinary protein loss, collagen deposition and associated nitroxidative stress while suppressing PRC and increasing renal mas and nNOS protein expression.

A large body of evidence suggests that dietary salt excess promotes renal injury in hypertensive subjects [2, 42, 43], although the underlying mechanisms are still not well defined. Increased blood pressure may be implicated in the detrimental renal effects of high salt diet, and we pos-

ited that nebivolol could decrease the hemodynamic burden imposed by high salt diet [44]. However, in this study nebivolol did not prevent the salt-induced increase in arterial pressure and did not increase serum NO metabolites. These data suggest that in this experimental model nebivolol did not facilitate systemic vascular NO production. However, in agreement with the concept that detrimental renal effects of dietary salt excess are partly mediated by non-pressure related mechanisms, nebivolol ameliorated salt-related renal functional and structural derangements.

The RAS have been also implicated in the harmful renal effects of high salt diet [7, 12]. The observed increased PRC in the present study confirmed our previous findings on paradoxical activation of RAS in salt-loaded rats [9]. In agreement, AT₁ receptor-blocking agents improved renal hemodynamics and function, reduced excessive proteinuria and fibrosis and ameliorated progressive proliferative glomerular changes in salt-sensitive hypertension [7, 43, 45]. Consistent with its β₁ receptor antagonism, nebivolol prevented renin release in rats fed the high salt diet. Further, reduced renal mas protein expression in untreated SHR fed high salt diet was associated with unfavorable changes in RBF and GFR and extensive fibrosis and proteinuria; nebivolol prevented the salt-induced reduction in mas expression along with improvement in RBF, urinary protein excretion, and collagen deposition but not reduced GFR. The critical role of Ang-(1-7) in the regulation of renal hemodynamics and tubuloglomerular structure and function is consistent with mas expression in afferent arterioles, glomerular

mesangial cells, and tubular epithelium [38, 46, 47]. Ang-(1-7) not only induced robust vasodilation in isolated renal afferent arterioles [48] but also abolished Ang II-induced renal vasoconstriction in isolated kidney from both Wistar-Kyoto rats and SHR [49]. Additionally, increases in circulating Ang-(1-7) by acute infusion or chronically in transgenic rats expressing an Ang-(1-7) producing fusion protein did not affect blood pressure but profoundly decreased renal vascular resistance [50, 51]; the effect was partially abolished by the specific mas antagonist [D-Ala⁷]-Ang-(1-7) (A-779) [51]. Moreover, mas-deficient mice developed extensive tubulointerstitial and glomerular fibrosis as well as renal dysfunction [37]. Consistent with these reports, results of the current investigation suggest deficient mas-mediated renal effects of Ang-(1-7) in response to high salt intake that was corrected by nebivolol treatment. The inability of nebivolol to prevent the salt-related reduction in GFR may be attributed to the lack of its antihypertensive action and corresponding persistent structural changes in glomerular capillary basement membranes. Additionally, it is not clear from this study how nebivolol regulates mas expression. In view of the recent report revealing a direct interaction between β -adrenergic and AT₁ receptors [52], further studies are warranted to explore the potential interaction between adrenergic and mas receptors as well.

This investigation assessed the expression of three NOS isoforms in order to elucidate the effects of dietary salt excess on NO system in SHR keeping in mind the important role of NO in regulation of RBF and growth of vascular smooth muscle and mesangial cells [26, 53, 54]. Interestingly, we demonstrated that the cortical eNOS expression was increased in salt-loaded SHR while there were no changes in the expression of nNOS. Although we did not determine directly the renal NOS-dependent production of NO, cortical NO_x levels paralleled the changes in the eNOS expression, suggesting a greater eNOS activity. The enhanced activity of NO system in response to high salt diet may be compensatory in order to prevent the excessive renal vasoconstriction and growth-promoting effects of activated sympathetic nervous system and RAS.

On the other hand, the functionality of the NO system depends not only on the NO production but also on its bioavailability that may be decreased due to enhanced superoxide formation. Increased oxidative stress, detrimental in many forms of salt-sensitive hypertension [11, 16, 55-57], is frequently associated with increased expression or activity of NADPH oxidase as the primary source of superoxide in the kidneys [11]. Thus, increased renal

superoxide level in response to high salt intake may react with locally produced NO diminishing bioavailability of NO while producing peroxynitrite [58] that may also contribute to renal injury [59, 60]. Indeed, in salt-loaded rats we demonstrated an enhanced 3-NT staining associated with increased expression of gp91phox, one of the critical components of NADPH oxidase, indicative of this enhanced interaction between NO and superoxide anion and subsequent peroxynitrite reaction with tyrosine residues on proteins. Increased eNOS expression may reflect the low levels of bioactive NO and its diminished negative regulatory role on eNOS [61, 62]. Alternatively, increased oxidative stress may promote oxidizing of tetrahydrobiopterin, a critical cofactor for the NOS, which becomes uncoupled and produces itself superoxide instead of NO [63]. However, this possibility seems less likely since increased renal NO_x paralleled the increase in renal eNOS expression mirroring NO formation rather than deficiency. These results imply that, despite the beneficial effects of NO on renal hemodynamics and function, high NO formation is not necessarily always advantageous for optimal renal protection. Importantly, nebivolol prevented excessive formation of peroxynitrite reflective in reduced 3-NT staining, gp91phox, and eNOS expression. Unlike other conventional β -blockers, nebivolol has strong anti-oxidative properties [33, 34, 64], and treatment with nebivolol decreased renal fibrosis and glomerular injury and improved endothelial dysfunction associated with increased NO bioavailability in different experimental models [15, 16, 65-67]. Indeed, in addition to the effects on the enzymatic source of superoxide and its intrinsic direct superoxide scavenger properties [34], nebivolol may have reduced oxidative injury in salt-loaded SHR by reducing the renal effects of Ang II while improving Ang-(1-7) signaling. Evidence from the literature testified for pro-oxidative action of Ang II [68], while recent studies revealed a potential of Ang-(1-7) to reduce NADPH oxidase activity in diabetic SHR kidney [35]. Moreover, Ang-(1-7) buffered the Ang II-induced superoxide production in nuclei isolated from the sheep kidneys [36]. Further studies are needed to elucidate the underlying mechanism of renal protection of nebivolol with respect to its potential interaction with mas signaling.

Finally, nebivolol-induced increase in nNOS expression may also contribute to its beneficial renal effects against salt-induced injury. Previous reports showed that the neuronal isoform could be detected in macula densa and arterioles as well as interlobular arteries [69-71], reflecting its input in regulating renal hemodynamics [72, 73]. Thus, it is plausible that NO derived from nNOS may

mediate renoprotective effects of nebivolol to prevent excessive renal vasoconstriction. To our knowledge, this is the first report on the effects of nebivolol on renal nNOS, although the relationship between β_3 adrenergic receptor and nNOS has been explored in the heart [74]. Nebivolol has additional β_3 -adrenergic receptor agonist effects, but the exact mechanisms as to how nebivolol regulates renal nNOS expression and its role in mediating renoprotection under the condition of dietary salt excess remain to be explored in future studies.

In summary, these studies showed that nebivolol, when given concomitantly with dietary salt excess, prevented kidney injury in SHR through a blood pressure-independent mechanism that may be related to reduction in oxidative stress, increased nNOS, and restoration of AT_1 /mas receptor balance.

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Disclosure Statement

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