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Original Contribution

Nebivolol attenuates prooxidant and profibrotic mechanisms involving TGF-β and MMPs, and decreases vascular remodeling in renovascular hypertension

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ABSTRACT

Nebivolol and metoprolol are β_1-adrenergic receptor blockers with different properties. We hypothesized that nebivolol, but not metoprolol, could attenuate prooxidant and profibrotic mechanisms of hypertension and therefore protect against the vascular remodeling associated with hypertension. Hypertension was induced in male Wistar rats by clipping the left renal artery. Six weeks after surgery, hypertensive and sham rats were treated with nebivolol (10 mg kg^{-1} day^{-1}) or metoprolol (20 mg kg^{-1} day^{-1}) for 4 weeks. Systolic blood pressure was monitored weekly. Morphologic changes in the aortic wall were studied in hematoxylin/eosin and picrosirius red sections. Aortic NAD(P)H activity and superoxide production were evaluated by luminescence and dihydroethidium, respectively, and TBARS levels were measured in plasma. Aortic nitrotyrosine staining was evaluated to assess peroxynitrite formation. TGF-β levels and p-ERK 1/2 expression were determined by immunofluorescence and Western blotting, respectively. Matrix metalloproteinase (MMP) activity and expression were determined by in situ zymography, gel zymography, Western blotting, and immunofluorescence, and TIMP-1 was assessed by immunohistochemistry. Both β_1-receptor antagonists exerted very similar antihypertensive effects. However, while metoprolol had no significant effects, nebivolol significantly attenuated vascular remodeling and collagen deposition associated with hypertension. Moreover, nebivolol, but not metoprolol, attenuated hypertension-induced increases in aortic NAD(P)H oxidase activity, superoxide production, TBARS concentrations, nitrotyrosine levels, TGF-β upregulation, and MMP-2 and -9 expression/activity. No effects on p-ERK 1/2 and TIMP-1 expression were found. These results show for the first time that nebivolol, but not metoprolol, attenuates prooxidant and profibrotic mechanisms involving TGF-β and MMP-2 and MMP-9, which promote vascular remodeling in hypertension.

Introduction

Hypertension promotes vascular remodeling associated with exposure of the vascular wall to chronic increases in blood pressure [1,2]. Indeed, sustained increases in blood pressure lead to collagen and elastin fragmentation and disrupt extracellular matrix (ECM) organization of conductance vessels. This process involves multiple mechanisms, leading to vascular cell proliferation, migration, and abnormal ECM synthesis and deposition [1–3]. In this respect, growing evidence implicates imbalanced matrix metalloproteinase (MMP) activity in experimental hypertension-induced vascular remodeling, especially MMP-2 [4–9]. Supporting these animal studies, clinical findings also implicate abnormal MMP activity in hypertension [10–12]. Adding complexity, a new intracellular isoform of MMP-2 has been recently described, and it is involved in cardiovascular disorders [13].

MMPs are critically regulated at posttranslational levels by proteolytic cleavage by other MMPs or oxidative and/or nitrosative stress [14,15]. Drugs with antioxidant properties attenuate hypertension-induced vascular remodeling, possibly as a result of less MMP activation [7,16–18]. However, antioxidants can also decrease hypertension-induced vascular remodeling by attenuating MMP-induced activation of profibrotic factors clearly involved in this process, including transforming grown factor (TGF)-β

Abbreviations: DHE, dihydroethidium; ECM, extracellular matrix; ERK 1/2, extracellular regulated kinase 1/2; 2K1C, 2 kidney, one clip; MMP, matrix metalloproteinase; SBP, systolic blood pressure; TGF, transforming grown factor; TBARS, thiobarbituric acid-reactive substances

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[19,20]. Moreover, antioxidants can directly inhibit hypertension-induced activation of extracellular regulated kinase 1/2 (ERK 1/2) [21], which is another profibrotic factor contributing to vascular remodeling of hypertension [2]. While lowering blood pressure clearly has a major impact on hypertension-induced vascular alterations, antihypertensive drugs with antioxidant properties may offer protection against the vascular alterations caused by hypertension that adds to their effects of blood pressure.

In this study, we compared the effects of two β_1-adrenergic receptor blockers (nebivolol and metoprolol) on the vascular alterations caused by 2 kidney, one clip (2K1C) hypertension. Our group has consistently shown increased oxidative stress and MMP activity in this animal model of hypertension, and inhibition of these alterations fully prevented vascular remodeling, even though blood pressure remained increased [5,16,22]. Our previous studies suggest that oxidative stress and imbalanced MMP activity play an important role in the vascular alterations of hypertension, independently of blood pressure [5,16,22]. Therefore, because nebivolol, but not metoprolol, has antioxidant properties [23–26], we hypothesized that nebivolol, but not metoprolol, could decrease NAD(P)H oxidase activation in the vasculature from 2K1C hypertensive rats and attenuate MMP activation, and profibrotic alterations associated with vascular remodeling. Our findings may offer a mechanistic insight to clinical [27] and experimental [28] studies showing reduced arterial stiffness in association with nebivolol, but not metoprolol.

Materials and methods

Animals and treatments

The study complied with guidelines of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, and the animals were handled according to the guiding principles published by the National Institutes of Health. Male Wistar rats (180–200 g) obtained from the colony at University of São Paulo were maintained on 12-h light/dark cycle at 25 °C with free access to rat chow and water. 2K1C hypertension was induced as previously described [29]. A silver clip (0.2 mm) was placed in the left renal artery of rats under anesthesia with ketamine 100 mg/kg and xylazine 10 mg/kg ip. Sham-operated rats underwent the same surgical procedure except for the clip placement. Tail systolic blood pressure (SBP) was assessed weekly by tail-cuff plethysmography.

Treatments were started 6 weeks after surgery and maintained for 4 weeks. The animals were randomly assigned to one of 6 groups: 2K1C or sham animals that received vehicle (ethanol 2% v/v), 2K1C and sham animals that received metoprolol (Meto) 20 mg kg^{-1} day^{-1} (Selozok; AstraZeneca), and 2K1C and sham animals that received nebivolol (Nebi) 10 mg kg^{-1} day^{-1} (Nebilet Biolab). The drugs were given by gavage, and the doses were chosen on the basis of previous studies using the same doses of both β-blockers, which produced similar effects [26,30]. At the end of the experimental period, animals were anesthetized as described above and killed by decapitation. Arterial blood samples were centrifuged at 1000*g* for 10 min and plasma fractions were immediately stored at −70 °C until used for biochemical measurements. Their thoracic aorta was removed and stored at −70 °C.

Morphometric analysis and composition of the vascular wall

Thoracic aortas were harvested, cleaned of connective tissue, fixed in 4% phosphate-buffered paraformaldehyde (Carlo Erba Reagents), pH 7.4, and embedded in paraffin blocks. Four-micrometer-thick slices were stained with hematoxylin (Fluka) and eosin (Fluka) (H&E) and picrosirius red (stain kit). Aortic media cross-sectional area (CSA) was calculated by subtracting the lumen internal area (A_i) from the external area (A_e) measured in tissue sections (50 ×) [5]. The external and internal diameters (ED and ID, respectively) were calculated as the square root of $4A_e/\pi$ (ED) and $4A_i/\pi$ (ID), respectively, and media thickness (M) was calculated as (ED–ID)/2. Finally, M to lumen diameter (M/L) was also calculated. The number of vascular smooth muscle cells (VSMCs) in the aortic media layer was measured by the tridimensional dissector method, which is independent of orientation, form, and size of the nucleus [5,22]. Picrosirius red staining was used to determine the collagen content in the aortic media layer with light microscopy (DMLB; Leica, Bensheim, Germany) and the image was captured at × 400. These structural analyses in the media were evaluated by using ImageJ Program (NIH–National Institutes of Health) as previously described [5,22].

Assessment of vascular ROS formation

NAD(P)H-dependent superoxide production was measured in aortic rings from rats. Aortic rings were transferred to luminescence vials containing 1 ml of Hanks buffer (Sigma), pH 7.4. After equilibration and background count, lucigenin (5 μmol L^{-1}; Sigma, M8010) and NAD(P)H (300 μmol L^{-1}; Sigma) were added to the vials and the luminescence count was measured continuously for 15 min in a Berthold FB12 single tube luminometer at 37 °C. Background signals from aortic rings were subtracted from the NAD(P)H-driven signals and the results were normalized by aortic dry weight and reported as relative luminescence units (RLU) mg^{-1} min^{-1}, as previously described [31].

Dihydroethidium (DHE; Sigma) was used to evaluate *in situ* production of ROS. Briefly, aortic tissues were vertically embedded in Tissue-tek. Aortic sections (5-μm-thick slices) were incubated with DHE 10 μmol L^{-1} (diluted in DMSO 0.01%; Sigma) for 30 min, and then washed 3 times with cold PBS (Sigma), pH 7.4, as previously described [31]. Sections were examined by fluorescence microscopy (Leica Imaging Systems Ltd., Cambridge, England) and the image was captured at × 400. Red fluorescence was measured using the ImageJ Program (National Institutes of Health). Red fluorescence intensity from 20 fields (corresponding to 20–30% of the total aortic area) selected around the vessel circumference was measured, and the arithmetic mean of the fluorescence from these fields was calculated for each slide.

Plasma lipid peroxide levels were determined by measuring thiobarbituric acid-reactive substances (TBARS) using a fluorimetric method that requires excitation at 515 nm and emission at 553 nm as previously described [32]. The lipoperoxide levels were expressed in terms of malondialdehyde (MDA) (nmol ml^{-1}).

Immunohistochemistry to assess nitrotyrosine and TIMP-1 expression

In order to assess nitrotyrosine and TIMP-1 (an endogenous tissue inhibitor of MMP) levels and location in the thoracic aorta, frozen aortic 5-μm sections were fixed in acetone and incubated with 3% H_2O_2 in water for 10 min at room temperature to block tissue peroxidase activity. Then tissue sections were incubated for 1 h with a specific rabbit anti-nitrotyrosine antibody (Millipore, USA) or mouse anti-TIMP-1 antibody (MAB3300, Chemicon) in dark humidified chambers. After washing 3 times, an anti-rabbit HRP-conjugated secondary antibody was added to the sections for 1 h at room temperature, and then washed 3 times with cold PBS. Positive staining appeared as a dark brown color visualized by adding 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen buffer for 20 min and washed 3 times with cold PBS (all solutions were from anti-rabbit poly horseradish peroxidase immunohistochemistry detection kit, Chemicon, USA, DAB-150).