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Original Contribution

β_1-Adrenergic blockers exert antioxidant effects, reduce matrix metalloproteinase activity, and improve renovascular hypertension-induced cardiac hypertrophy

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ABSTRACT

Hypertension induces left-ventricular hypertrophy (LVH) by mechanisms involving oxidative stress and unbalanced cardiac matrix metalloproteinase (MMP) activity. We hypothesized that β_1-adrenergic receptor blockers with antioxidant properties (nebivolol) could reverse hypertension-induced LVH more effectively than conventional β_1-blockers (metoprolol) when used at doses that exert similar antihypertensive effects. Two-kidney one-clip (2K1C) hypertension was induced in male Wistar rats. 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 by tail-cuff plethysmography. LV structural changes and fibrosis were studied in hematoxylin/eosin- and picrosirius-stained sections, respectively. Cardiac MMP levels and activity were determined by in situ zymography, gel zymography, and immunofluorescence. Dihydroethidium and lucigenin-derived chemiluminescence assays were used to assess cardiac reactive oxygen species (ROS) production. Nitrotyrosine levels were determined in LV samples by immunohistochemistry and green fluorescence and were evaluated using the ImageJ software. Cardiac protein kinase B/Akt (AKT) phosphorylation state was assessed by Western blot. Both β-blockers exerted similar antihypertensive effects and attenuated hypertension-induced cardiac remodeling. Both drugs reduced myocyte hypertrophy and collagen deposition in 2K1C rats. These effects were associated with lower cardiac ROS and nitrotyrosine levels and attenuation of hypertension-induced increases in cardiac MMP-2 levels and in situ gelatinolytic activity after treatment with both β-blockers. Whereas hypertension increased AKT phosphorylation, no effects were found with β-blockers. In conclusion, we found evidence that two β_1-blockers with different properties attenuate hypertension-induced LV hypertrophy and cardiac collagen deposition in association with significant cardiac antioxidant effects and MMP-2 downregulation, thus suggesting a critical role for β_1-adrenergic receptors in mediating those effects. Nebivolol is not superior to metoprolol, at least with respect to their capacity to reverse hypertension-induced LVH.

Hypertension is a major risk factor for cardiovascular events [1] at least in part attributable to cardiac remodeling associated with left-ventricular hypertrophy (LVH), which may lead to heart failure [2]. Despite the alterations triggered by chronic hypertension on cardiomyocytes, important modifications also occur in the cardiac extracellular matrix (ECM) integrity during the progression of LVH [3–5]. Intense collagen deposition promotes myocardial stiffening and impairs myocardial function in association with the cardiac hypertrophy of hypertension [6,7].

Matrix metalloproteinases (MMPs) are critically involved in the turnover of cardiac ECM, and unbalanced MMP activity usually results in fibrosis [8–10]. Innumerous studies have shown that, among other members, MMP-2 activity plays a key role in hypertensive cardiac remodeling and in other cardiovascular disorders [9,11–14]. This enzyme is activated under conditions that increase the formation of reactive oxygen species (ROS) [10,15]. Interestingly, recent evidence suggests that a particular N-terminal-truncated MMP-2 isoform is induced by oxidative stress [16] and promotes cardiomyocyte hypertrophy in the absence of superimposed injury [17]. This is important because

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increased ROS levels apparently are major activators of MMP-2 in hypertension [11,18–20], and antioxidant drugs reduced MMP activity and protected against hypertensive cardiac hypertrophy, particularly in experimental models with enhanced angiotensin II activity [11,19].

β-Adrenergic blockers are widely used in hypertensive patients. However, the mechanisms by which β-blockers may attenuate or prevent hypertensive cardiac hypertrophy have not been clearly defined. In this regard, nebivolol is a cardioselective β_1-adrenergic receptor antagonist that, in addition to its effects on β-adrenergic receptors, exerts antioxidants effects [20–23]. This particular property of nebivolol may explain why this β-blocker has been considered superior to other β-blockers such as metoprolol after myocardial infarction [21] or to prevent the vascular profibrotic alterations and remodeling associated with hypertension [20]. However, it is not clear whether antioxidant effects and the apparent superiority of nebivolol compared to metoprolol are consistently found. In this study, we hypothesized that treatment with either nebivolol or metoprolol could attenuate hypertension-induced cardiac oxidative stress, MMP-2 activation, and LVH at therapeutic doses that reduce blood pressure. Whereas antioxidants effects have been ascribed to metoprolol in some recent studies [24–26], it is not clear whether metoprolol and nebivolol have antioxidant effects at therapeutic doses. This is a very important issue because many drugs with putative antioxidant effects require high concentration or show antioxidant effects only under in vitro conditions.

Materials and methods

Animals and treatments

The study complied with the 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 the University of São Paulo were maintained on a 12-h light/dark cycle at 25 °C with free access to rat chow and water. Two-kidney one-clip (2K1C) hypertension was induced as previously described [10]. 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 six groups: 2K1C or sham animals that received vehicle (ethanol 2% v/v), 2K1C and sham animals that received metoprolol (Meto) at 20 mg kg^{-1} day^{-1} (Selozok; AstraZeneca), and 2K1C and sham animals that received nebivolol (Nebi) at 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 antihypertensive effects [20,21,27].

Harvesting of the hearts and histological analysis

The animals were weighed and anesthetized, and the thoracic cavity was opened to expose the still-beating heart. The hearts were rapidly removed, rinsed in ice-cold 0.9% saline solution, blotted, weighed, and fixed as a whole in phosphate-buffered 10% formalin (pH 7.3) for histological study. Both ventricles from each heart were isolated and cut into two fragments by a midventricular coronal section. Each block was serially cut in the same direction and 4-μm-thick sections were stained with hematoxylin and eosin. The left ventricular wall and septum thickness and the area corresponding to the left ventricular chamber were determined in the first three histological sections of each block. Morphometrics analyses were carried out with ImageJ software developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image. Average myocyte diameter was determined from 20 measurements in each ventricle, which were made at a magnification of 400× in longitudinally oriented myofibers. Myocyte diameter was obtained in the region of the nucleus of those cells in which the nuclear envelope was sharply defined at both ends, which corresponds to the nucleus length and to its position at an equal distance from the sides of the myocytes. The measurements were done with the Leica Qwin software (Leica Imaging Systems Ltd, Cambridge, UK) in conjunction with a Leica microscope (Leica DMR, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany), videocamera, and online computer. To estimate the collagen area (%) of fibrosis in picrosirius red-stained sections, quantitative examination of the left ventricular and septum myocardium was carried out on a medium-power light-microscopic field (×400). For each heart, 15 fields per region per rat were randomly selected and analyzed using Leica Qwin software (Leica Imaging Systems). The mean value was subsequently calculated.

Assessment of cardiac ROS and nitrotyrosine levels

Dihydroethidium (DHE; Sigma) was used to evaluate in situ production of ROS [28,29]. Briefly, the heart tissue was embedded in Tissue-Tek. Left ventricle sections (5-μm-thick slices) were incubated in 10 μmol/L DHE (diluted in 0.01% dimethyl sulfoxide; Sigma) for 30 min and then washed three times with cold phosphate-buffered saline (PBS; Sigma), pH 7.4, as previously described [10]. Sections were examined by confocal microscopy (Leica Model SPE, Leica Imaging Systems Ltd) using λ_{ex} 405 nm laser excitation [28]. The image was captured at ×400 and red fluorescence was measured using the ImageJ Program (National Institutes of Health). Some experiments were performed after preincubation for 1 h with diphenyliodonium (DPI; 100 μmol/L; an inhibitor of flavoproteins), tiron (1 mmol/L; a superoxide scavenger), or superoxide dismutase conjugated with polyethylene glycol (PEG–SOD; 200 U/ml).

Lucigenin-derived chemiluminescence assay was also utilized to evaluate superoxide anion production, as previously described, with little modification [30]. Briefly, left ventricle homogenates were prepared in phosphate buffer, pH 7.4 (KH_2PO_4 50 mM, EDTA 1 mM, sucrose 150 mM) and immediately transferred (50 μl) to the luminescence microplate, and 175 μl of phosphate buffer with 5 μmol/L lucigenin was added. After background measurement, 300 μM NADPH was added to reaction (final volume of 250 μl). Luminescence was measured at 37 °C every second for 15 min in a luminometer (Orion II Luminometer, Berthold). Background signals from samples were subtracted from the NADPH-driven signals and the results were normalized by left ventricle dry weight and reported as relative luminescence units (RLU)/mg.

To assess nitrotyrosine formation in the heart, 5-μm-thick frozen 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 the tissue sections were incubated for 1 h with a specific rabbit anti-nitrotyrosine antibody (Millipore, USA) in dark humidified chambers. The samples were washed three times, and an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody was added to the sections for 1 h at room temperature and then washed three times with cold PBS. Positive staining appeared as a dark brown color visualized by adding 3,3′-diaminobenzidine tetrahydrochloride chromogen

buffer for 20 min followed by three washes with cold PBS (all solutions were from an anti-rabbit poly-horseradish peroxidase immunohistochemistry detection kit, Chemicon, USA, DAB-150). Sections were counterstained with hematoxylin and examined by optic microscopy (Leica Imaging Systems Ltd) and the image was captured at ×400. The amounts of nitrotyrosine in stained sections were obtained by quantification of the immunoreactivity shown as brown color intensity and examined by two skilled blinded observers. The evaluation of immunoreactivity was scored qualitatively and quantitatively from 1 (low) to 5 (strong). Each score reflects changes in the intensity of staining.

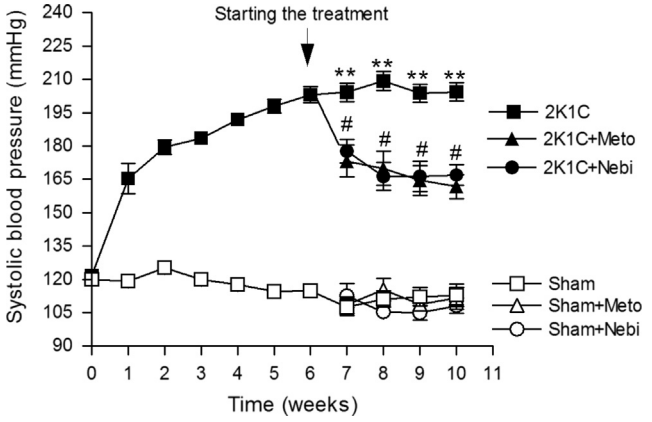

Fig. 1. Systolic blood pressure (mm Hg) measured by the tail-cuff method before and after treatment with vehicle (control), metoprolol, or nebivolol. Data are shown as the mean ± SEM (n = 11–14/group). $^{**}P < 0.05$ vs the 2K1C + Nebi or the 2K1C + Meto group. $^{\#}P < 0.05$ vs the respective sham group.

Measurement of cardiac MMP-2 levels by gelatin zymography

Gelatin zymography of MMP-2 was used to measure MMP-2 levels in left ventricle (LV) samples as previously described [11,12]. Briefly, frozen LV samples (five per group) were homogenized in ice-cold RIPA buffer (500 μl for each 0.1 g of LV sample) containing 1 mM 1,10-*ortho*-phenanthroline, 1 mM phenylmethanesulfonyl fluoride, and 1 mM *N*-ethylmaleimide. The samples were placed on ice within a refrigerator for 30 min with gentle stirring and then centrifuged at 10,000 *g* for 15 min. The protein concentrations were measured using the Bradford method (Sigma). Thereafter, 60 μg of protein was diluted 1:1 with sample buffer (final concentration: 2% SDS, 125 mM Tris–HCl, pH 6.8, 10% glycerol, and 0.001% bromophenol blue) and subjected to electrophoresis on 12% SDS–polyacrylamide gel electrophoresis copolymerized with gelatin (0.05%) as the substrate. After electrophoresis was completed, the gel was incubated for 1 h at room temperature in a 2% Triton X-100 solution, washed, and incubated at 37 °C overnight in Tris–HCl buffer, pH 7.4, containing 10 mmol/L $CaCl_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activity was detected as an unstained band against the background of Coomassie blue-stained gelatin. Enzyme activity was assayed by densitometry using ImageJ software. The MMP-2 forms were identified as bands at 75, 72, and 64 kDa.

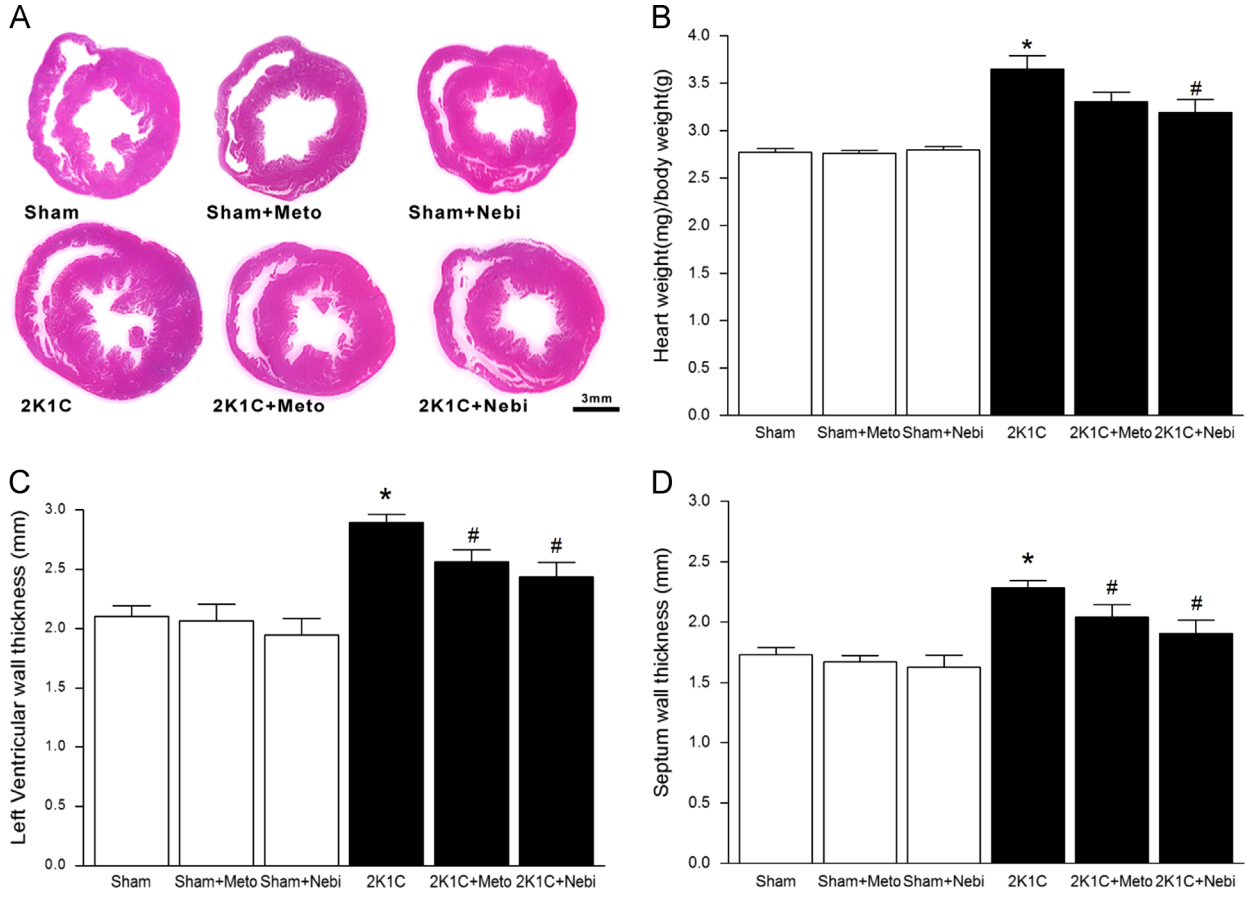

Fig. 2. Effects of treatment with β-blockers on hypertension-induced cardiac remodeling. (A) Representative photomicrographs of transverse heart sections used to determine left ventricle area. (B) Heart weight/body ratio. (C) Left ventricle wall thickness. (D) Septum wall thickness. Data are shown as the mean ± SEM (n = 5 or 6/group). $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the 2K1C group.

in situ zymography and immunofluorescence to assess gelatinolytic activity and MMP-2 expression

in situ MMP activity was measured in frozen LV using DQ gelatin (E12055, Molecular Probes, Eugene, OR, USA) as a fluorogenic substrate [31]. Briefly, LV samples were embedded in Tissue-Tek and cut into 5-μm sections with a cryostat. Sample sections were incubated with 1.0 mg/ml DQ gelatin in Tris–$CaCl_2$ buffer (50 mM Tris, 10 mM $CaCl_2$, 1 mM $ZnCl_2$) in dark humidified chambers for 1 h. The sections were examined with fluorescence microscopy (Leica Imaging Systems Ltd) and the image was captured at a magnification of 400 ×. Proteolytic activity was detected as bright green fluorescence, which indicates substrate breakdown, and was evaluated using the ImageJ Program (National Institutes of Health).

To evaluate MMP-2 expression, 5-μm tissue sections were incubated with mouse anti-MMP-2 monoclonal antibody (MAB3308, Chemicon International, Temecula, CA, USA) for 1 h in a dark humidified chamber (at a 1:1000 dilution) [11]. Green fluorescence was visualized by adding a fluorescein-conjugated anti-mouse secondary antibody (1:200; FI 2000, Vector Laboratories, USA) for 1 h. To confirm the specificity of antibodies, the primary antibody was omitted and substituted by PBS 1% bovine serum albumin (BSA). Fluorescein did not bind nonspecifically to the tissue sections. MMP-2 expression was detected as bright green fluorescence and was evaluated using the ImageJ software.

Western blot analysis of protein kinase B/Akt (AKT)

AKT phosphorylation state was assessed in the rat hearts because increases in the basal phosphorylation state of AKT promote cardiac hypertrophy [32]. Moreover, AKT has been shown to mediate hypertrophic effects associated with stimulation of β-adrenergic receptors [33]. Therefore, examining the effects of β-adrenergic blockers on AKT phosphorylation state could offer additional mechanistic insight to this study.

Briefly, heart extracts were homogenized in cold RIPA buffer. Forty micrograms of protein extracts was separated by SDS–PAGE using a12% polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes (GE Healthcare, Madison, WI, USA). After being blocked in 5% BSA, membranes were incubated overnight at 4 °C with primary antibody directed against phospho-AKT (Ser473) or total AKT (1:1000; Cell Signaling). Then the membranes were incubated with HRP-labeled secondary goat anti-rabbit antibody (1:1000; Millipore) and revealed with an ECL chemiluminescence kit (GE Healthcare).

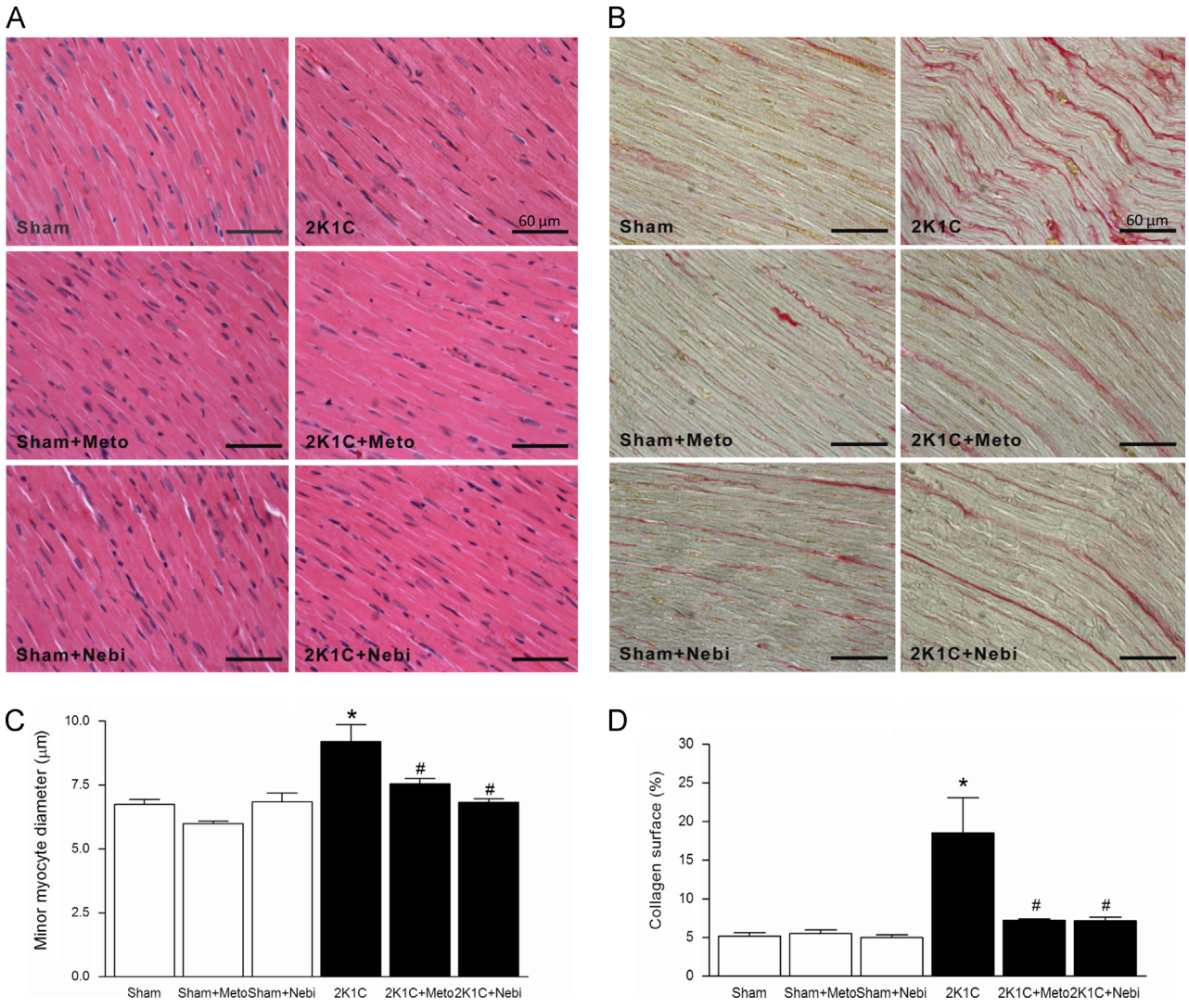

Fig. 3. Effects of treatment with β-blockers on myocyte diameter and collagen area. (A) Representative photomicrographs of sections from left ventricle myocytes (original magnification 400 ×). (B) Representative photomicrographs of sections from left ventricle stained with picrosirius red (original magnification 400 ×). (C) Values of minor diameter of myocytes. (D) Values of collagen surface area (%) in the left ventricle and septum. Data are shown as the mean ± SEM (n = 5–7/group). $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the 2K1C group.

Statistical analysis

The results are expressed as means ± SEM. Between-group comparisons were carried out with two- or one-way analysis of variance followed by the Tukey test. A probability value <0.05 was considered significant.

Results

The two β-blockers exerted similar antihypertensive effects

SBP increased from baseline (120 mm Hg) to approximately 200 mm Hg in 2K1C hypertensive rats, whereas no significant

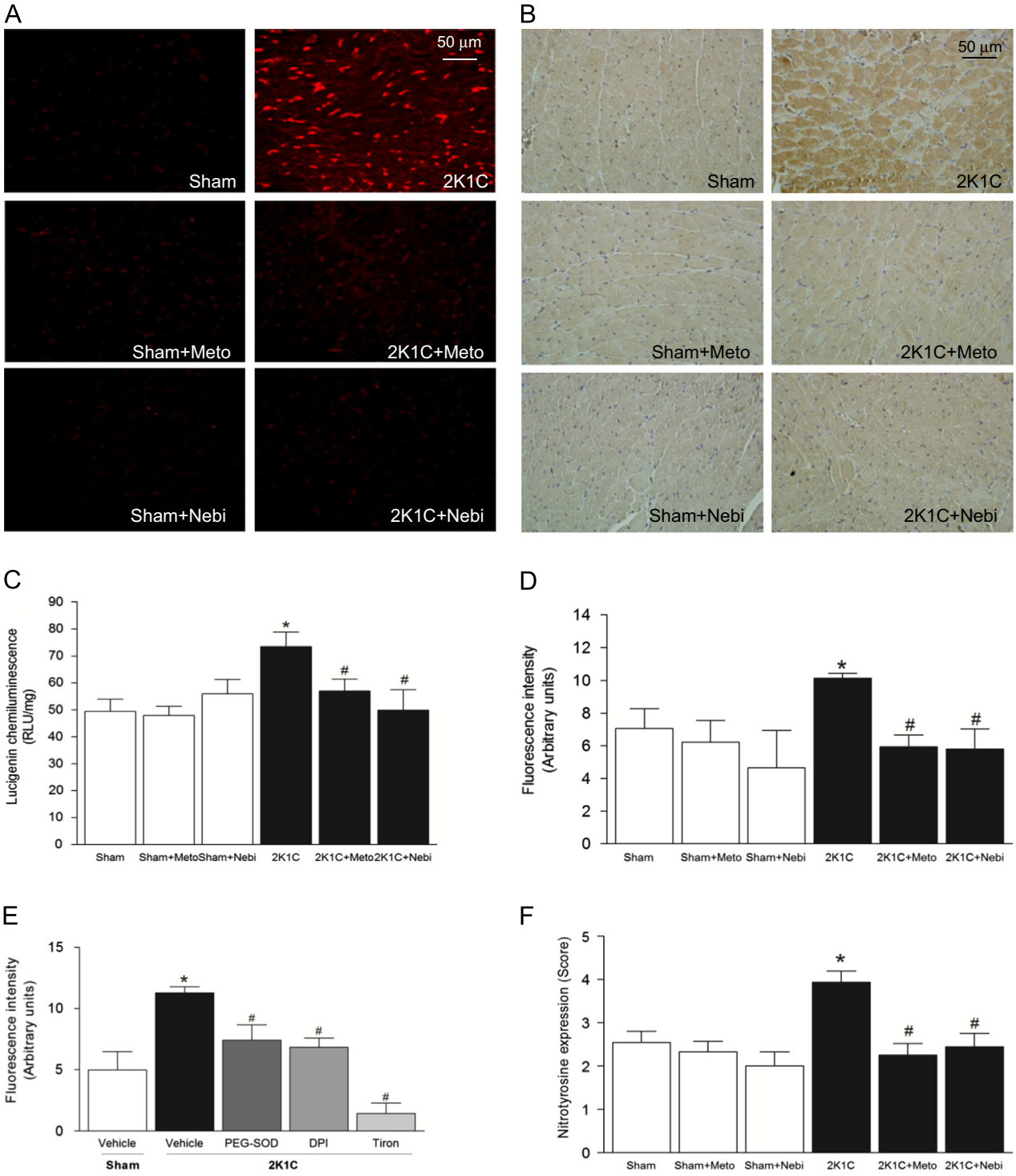

Fig. 4. (A) Representative confocal photomicrographs (original magnification 400 ×) of cardiac sections incubated with DHE. Red fluorescence represents reactive oxygen species production. (B) Representative photomicrographs of nitrotyrosine immunostaining performed in left-ventricular cryosections (original magnification 400 ×). (C) Quantification of left-ventricular lucigenin chemiluminescence. (D) Quantification of left-ventricular fluorescence of DHE by confocal microscopy using λ_{ex} 405 nm laser excitation. (E) Results for positive controls. This graph shows increased DHE oxidation in 2K1C hypertension, which decreased with preincubation for 1 h with 200 U/ml PEG–SOD, or 100 µmol/L DPI, or 1 mmol/L tiron. (F) Quantification of left-ventricular nitrotyrosine expression. Data are shown as the mean ± SEM (n = 5–7/group). $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the 2K1C group.

changes were found in the sham-operated group ($P < 0.05$; Fig. 1). Treatment with drugs was started after 6 weeks of hypertension, and similar decreases in SBP were found in 2K1C animals treated with nebivolol or with metoprolol (from approximately 200 to 162 ± 19 and 167 ± 17 mm Hg, respectively; $P < 0.05$; Fig. 1). No significant changes were found in sham-operated animals.

Both β-blockers attenuated hypertension-induced cardiac remodeling

Representative photomicrographs of transverse heart sections are shown in Fig. 2A. Hypertension increased all parameters reflecting cardiac hypertrophy, including heart weight/body weight ratio, LV wall thickness, and septum wall thickness, and treatment with either metroprolol or nebivolol attenuated hypertension-induced alterations in these (all $P < 0.05$; Fig. 2B–D, respectively), except for heart weight/body weight ratio in animals treated with metoprolol, which only tended to decrease with this treatment ($P > 0.05$; Fig. 2B). No significant changes were found in sham-operated animals treated with drugs.

Both β-blockers attenuated myocyte hypertrophy and profibrotic alterations associated with hypertension

The minor diameter of myocytes and fibrosis in LV sections were evaluated to assess further alterations associated with hypertension, and representative photomicrographs are shown in Fig. 3A and B, respectively. As expected, hypertension induced significant myocyte hypertrophy and fibrosis in the 2K1C group compared to the sham group, and treatment with both metoprolol and nebivolol reduced myocyte hypertrophy and collagen deposition in 2K1C rats ($P < 0.05$ vs untreated 2K1C group; Fig. 3C and D). No significant changes were observed in the sham-operated groups.

Both β-blockers attenuated cardiac oxidative and nitrosative stress associated with hypertension

Fig. 4A and B show representative confocal photomicrographs of ROS detection by DHE and nitrotyrosine expression in LV from rats, respectively. To further validate oxidative stress assessed by DHE, ROS formation was also studied using a lucigenin assay (Fig. 4C). Hypertension consistently increased cardiac ROS levels

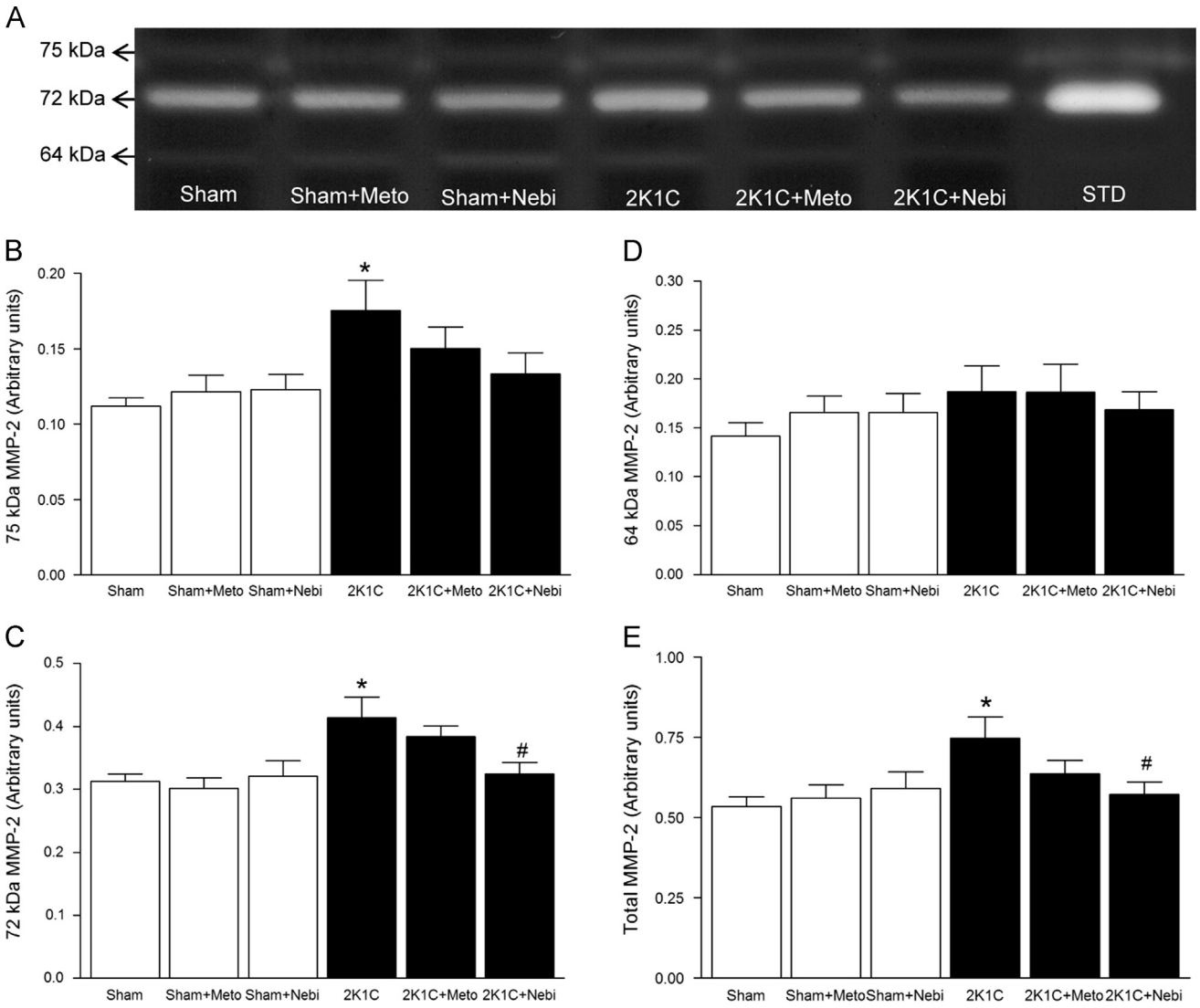

Fig. 5. (A) Representative SDS–PAGE gelatin zymogram of left ventricle samples. Values for (B) 75-kDa MMP-2, (C) 72-kDa MMP-2, and (D) 64-kDa MMP-2 isoforms and (E) total MMP-2 (75 + 72 + 64 kDa MMP-2) are shown. Data are shown as the mean ± SEM ($n = 11$ or 12/group). $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the 2K1C group.

detected by DHE (Fig. 4A and D), nitrotyrosine expression (Fig. 4B and E), and lucigenin-derived chemiluminescence (Fig. 4C) compared to the sham-operated group (all $P < 0.05$). Interestingly, treatment with both β-blockers significantly attenuated all those alterations associated with hypertension (all $P < 0.05$; Fig. 4A–D, and F). Fig. 4E shows the results for positive controls. The increases in DHE oxidation found in 2K1C hypertension were attenuated after preincubation for 1 h with PEG–SOD, DPI, or tiron (all $P < 0.05$; Fig. 4E).

Whereas nebivolol attenuated hypertension-induced increases in cardiac MMP-2 levels, metoprolol only tended to exert such an effect

A representative zymogram of cardiac extracts is shown in Fig. 5A, which shows three bands corresponding to three isoforms of MMP-2: 75, 72, and 64 kDa. Hypertension increased the cardiac levels of both the 75- and the 72-kDa MMP-2 isoform, but not the 64-kDa isoform, compared to the sham-operated group (Fig. 5B–D, respectively), thus resulting in increased total MMP-2 ($P < 0.05$; Fig. 5E). Treatment with nebivolol attenuated hypertension-induced increases in different MMP-2 isoforms, thus lowering total MMP-2 levels ($P < 0.05$; Fig. 5B, C, and E). In contrast to the effects of nebivolol, treatment with metoprolol tended to decrease cardiac MMP-2 levels, but no significant effects were found (all $P > 0.05$; Fig. 5B–E).

Both β-blockers attenuated hypertension-induced increases in cardiac in situ gelatinolytic activity and MMP-2 expression

To further improve our assessment of cardiac MMP activity and MMP-2 expression, we measured in situ gelatinolytic activity and assessed MMP-2 expression by immunofluorescence. Representative photomicrographs showing both assessments in cardiac samples are shown in Fig. 6A and B. Increased gelatinolytic activity and MMP-2 levels were found in hearts from 2K1C hypertensive rats compared to sham-operated controls ($P < 0.05$; Fig. 6C and D,

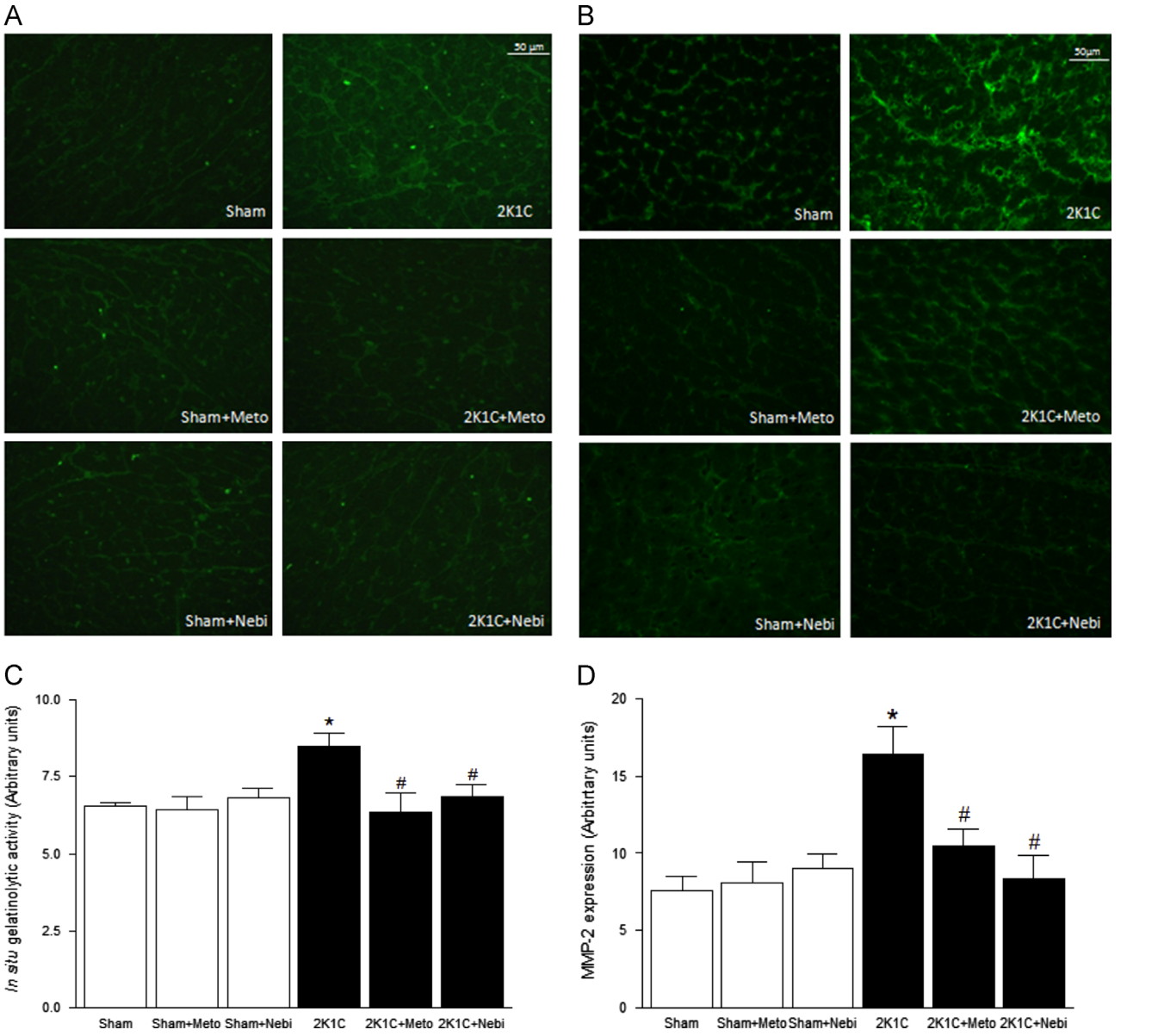

Fig. 6. in situ gelatinolytic activity and MMP-2 expression in the left ventricles from rats. (A) Representative photomicrographs of gelatinolytic activity and (B) MMP-2 staining in the left ventricles (original magnification 400 ×). (C) Quantification of left ventricle surface area covered by bright green fluorescence, which reflects gelatinolytic activity. (D) Green fluorescence intensity, which reflects MMP-2 expression in cardiac tissue. Values are shown as means ± SEM ($n = 4$–6/group). $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the 2K1C group.

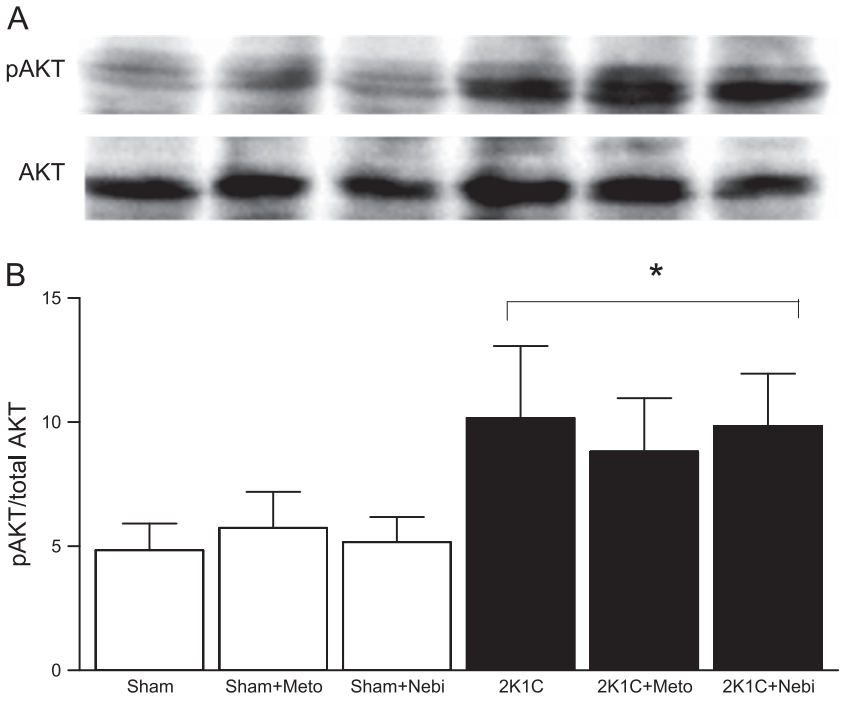

Fig. 7. Western blot analysis of protein kinase B/Akt (AKT) phosphorylation state. (A) Representative Western blot of phospho-AKT (Ser473) and total AKT. (B) Phospho-AKT-to-total AKT ratio, which reflects the state of AKT phosphorylation. Values are shown as means ± SEM ($n = 6$/group). *$P < 0.05$ vs the sham groups.

respectively). Treatment with nebivolol or metoprolol almost completely blunted hypertension-induced increases in both in situ gelatinolytic activity and MMP-2 expression in the hearts from hypertensive animals ($P < 0.05$; Fig. 6C and D, respectively).

Neither β-blocker affected AKT phosphorylation state

Increased AKT phosphorylation is a critical mechanism leading to cardiomyocyte and cardiac hypertrophy [32], which could be reversed by therapy with β-blockers [33]. Although we found significant increases in the levels of phospho-AKT in hypertensive rats ($P < 0.05$; Fig. 7A and B), treatment with β-blockers did not affect this alteration.

Discussion

This is the first study to report that both nebivolol and metoprolol used at doses that resulted in similar antihypertensive effects can equally reverse hypertensive cardiomyocyte and LV hypertrophy in association with antioxidant effects and lower LV MMP activity.

Previous studies have compared the effects of nebivolol and metoprolol under different conditions [20–22], and most studies showed that nebivolol is superior to metoprolol in protecting against the activation of pathophysiological mechanisms promoting cardiovascular dysfunction. For example, nebivolol, but not metoprolol, blunted cardiac NADPH oxidase activation after myocardial infarction, improved LV dysfunction, and inhibited cardiomyocyte hypertrophy found during this condition [21]. In line with those findings, nebivolol, but not metoprolol, attenuated the vascular remodeling associated with increased oxidative stress and MMP activation in hypertensive rats [20]. Therefore, at least some studies support the idea that nebivolol induces effects beyond those usually found with conventional β_1-blockers [20,21]. However, our present results suggest that this idea is probably not valid in the particular setting of experimental hypertensive LV hypertrophy. Indeed, we studied different β-blockers that apparently have different pharmacological properties but exert similar protective effects. This important observation suggests that blocking β-adrenergic receptors is the main reason explaining how β-blockers reverse hypertensive LV hypertrophy.

Transgenic mice overexpressing β_1-adrenergic receptors showed increased MMP-2 levels and enhanced cardiac remodeling [34]. Whereas activation of β-adrenergic receptors upregulates MMPs, both in vivo and in vitro [34–37], little is known about the effects of β-blockers on MMP levels and activity, and our results consistently show that treatment with β-blockers significantly blunts hypertension-induced increases in MMP-2 expression and activity, although the effects of metoprolol on MMP-2 levels assessed by zymography were slightly less important than those found with nebivolol. However, we used three different methods to assess MMP activity in the heart, and similar results were found with independent approaches, clearly showing that treatment with both β-blockers blunts 2K1C hypertension-induced increases in cardiac MMP-2 levels and activity.

Mechanistically, our findings suggest that both β-blockers tested here may have blunted β-adrenergic receptor-stimulated ROS formation and MMP-2 activation, and this effect may have had critical consequences in the present hypertension model. This suggestion is supported by previous studies showing that activation of β-adrenergic receptors increases ROS formation as a result of increased protein kinase C activity leading to subsequent NADPH oxidase activation [38–40]. Increased ROS levels, in turn, activate MMPs and promote cardiac hypertrophy [11,17,19]. In agreement with suggestion, we found increased ROS formation, lucigenin-derived chemiluminescence, and nitrotyrosine expression associated with augmented MMP-2 levels and activity in the hearts from 2K1C hypertensive animals, and all those alterations were significantly attenuated after treatment with both β_1-blockers, thus suggesting that blocking β-adrenergic receptors is critical to reverse hypertension-induced LV hypertrophy. Interestingly, although increased AKT phosphorylation has been shown to be an important mechanism underlying hypertrophic modifications [32], potentially

dependent on the stimulation of β-adrenergic receptors [33], our results suggest that this mechanism is not relevant to explain the pharmacological effects shown here.

Although various MMPs are expressed in the myocardium, growing evidence indicates that MMP-2 plays a major role in cardiac remodeling [12,13,17]. Indeed, this particular MMP is found at the ECM and within cardiomyocytes, and unbalanced MMP-2 activity impairs ECM integrity and cardiomyocyte function [41,42]. Although increased MMP-2 activity promotes collagen degradation, there is also increased expression and activity of profibrotic factors resulting in collagen accumulation [11,43]. Consistent with previous studies, we found augmented collagen accumulation in the LV from hypertensive rats [5,10,44], and treatment with nebivolol or metoprolol was equally effective in decreasing hypertensive collagen deposition.

The methods used to assess ROS production in this study deserve some comment. There are some controversial issues with both DHE and lucigenin assays, particularly with respect to self-propagation reactions with the use of fluorescent probes such as DHE [28,29] and the possibility of redox cycling of lucigenin [45,46], which may limit their utility to assess superoxide formation, as previously discussed [28,29,45,46]. Although the main goal of this study was not to detect a specific oxidant, it is important to note that the results obtained with both assays were in full agreement with nitrotyrosine results. Importantly, we used confocal microscopy using excitation at 405 nm for the DHE assay, which minimizes possible artifacts [28]. Moreover, we used lucigenin at a 5 μmol/L concentration, which has been suggested as a non-redox-cycling concentration [47]. Therefore, our results may provide relevant information about overall oxidant production.

In conclusion, we found evidence that two β_1-blockers with different properties attenuate hypertension-induced LV hypertrophy and cardiac collagen deposition in association with significant cardiac antioxidant effects and MMP-2 downregulation. Because both β_1-blockers exerted similar antihypertensive effects, our results suggest a critical role for β_1-adrenergic receptors in mediating those effects. Clinical studies should be carried out to compare the effects of various β_1-blockers on hypertension-induced LV hypertrophy.

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