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Nebivolol for Improving Endothelial Dysfunction, Pulmonary Vascular Remodeling, and Right Heart Function in Pulmonary Hypertension

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ABSTRACT

BACKGROUND Endothelial cell (EC) dysfunction plays a central role in the pathogenesis of pulmonary arterial hypertension (PAH), promoting vasoconstriction, smooth muscle proliferation, and inflammation.

OBJECTIVES This study sought to test the hypothesis that nebivolol, a β_1-antagonist and $\beta_{2,3}$-agonist, may improve PAH and reverse the PAH-related phenotype of pulmonary ECs (P-EC).

METHODS We compared the effects of nebivolol with metoprolol, a first-generation β_1-selective β-blocker, on human cultured PAH and control P-EC proliferation, vasoactive and proinflammatory factor production, and crosstalk with PA smooth muscle cells. We assessed the effects of both β-blockers in precontracted PA rings. We also compared the effects of both β-blockers in experimental PAH.

RESULTS PAH P-ECs overexpressed the proinflammatory mediators interleukin-6 and monocyte chemoattractant protein-1, fibroblast growth factor-2, and the potent vasoconstrictive agent endothelin-1 as compared with control cells. This pathological phenotype was corrected by nebivolol but not metoprolol in a dose-dependent fashion. We confirmed that PAH P-EC proliferate more than control cells and stimulate more PA smooth muscle cell mitosis, a growth abnormality that was normalized by nebivolol but not by metoprolol. Nebivolol but not metoprolol induced endothelium-dependent and nitric oxide–dependent relaxation of PA. Nebivolol was more potent than metoprolol in improving cardiac function, pulmonary vascular remodeling, and inflammation of rats with monocrotaline-induced pulmonary hypertension.

CONCLUSIONS Nebivolol could be a promising option for the management of PAH, improving endothelial dysfunction, pulmonary vascular remodeling, and right heart function. Until clinical studies are undertaken, however, routine use of β-blockers in PAH cannot be recommended. (J Am Coll Cardiol 2015;65:668–80) © 2015 by the American College of Cardiology Foundation.

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The pathogenesis of pulmonary arterial hypertension (PAH) involves a complex and multifactorial process in which endothelial cell (EC) dysfunction appears to play an integral role in mediating the structural changes in the pulmonary vasculature (1). An altered production of various endothelial vasoactive mediators, such as nitric oxide (NO), prostacyclin, endothelin (ET)-1, serotonin, and thromboxane has been increasingly recognized in patients with PAH. Because most of these mediators affect the growth of smooth muscle cells, an alteration in their production by ECs may facilitate the development of pulmonary vascular hypertrophy and structural remodeling characteristic of PAH (1).

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Although efficacious vasodilatory therapies targeting endothelial dysfunction have been developed (2), the survival of patients with PAH remains unsatisfactory (55% at 3 years) (3). Recently, nonselective inhibitors of the platelet-derived growth factor receptor tyrosine kinase, such as imatinib, have been tested in PAH as anti-remodeling agents. The IMPRES (The Imatinib in Pulmonary Arterial Hypertension, a Randomized, Efficacy Study) revealed an added benefit for imatinib against a background of conventional combination therapy but highlighted severe side effects, such as subdural hematoma, questioning the use of this poorly tolerated drug in PAH (4). Hence, new therapeutic avenues are wanted.

Adrenergic receptors (ARs) represent major regulators of the cardiovascular system and of EC function in particular. Recent data revealed that sympathetic overstimulation is strongly related to mortality, and blockade of β-ARs in experimental pulmonary hypertension (PH) improved survival and cardiac function (5–8). Although current treatment guidelines still advise against the use of AR blockers in PAH because of a feared induction of systemic hypotension and reduction in exercise capacity, these promising preclinical studies have been followed by still-ongoing studies to test the safety and efficacy of carvedilol and bisoprolol in PAH patients. Given the role of the β_2-AR in endothelial function and the regulation of pulmonary vascular tone, we investigated the effect of nebivolol, a third-generation β-AR blocker, on EC dysfunction in PAH. Nebivolol is a β_1-antagonist and $\beta_{2,3}$-agonist and has direct vasodilator properties in addition to its adrenergic-blocking characteristics. Nebivolol has proven beneficial effects on pulmonary artery pressure, pulmonary wedge pressure, exercise capacity, and left ventricular ejection fraction in patients with left heart disease (9). Moreover, nebivolol is well-tolerated and highly effective in patients with chronic obstructive pulmonary disease in association with arterial hypertension (10).

In this study, we compared the effects of nebivolol with metoprolol, a first-generation β_1-selective β-blocker on cultured PAH and control pulmonary endothelial cell (P-EC) proliferation, vasoactive, and proinflammatory factors production and crosstalk with PA smooth muscle cells (PASMCs). We assessed the effects of both β-blockers in precontracted rat PA rings. We also compared the effects of both β-blockers in experimental PH in rats induced by monocrotaline (MCT).

ABBREVIATIONS AND ACRONYMS

AR = adrenergic receptor
EC = endothelial cell
ET = endothelin
IPAH = idiopathic pulmonary arterial hypertension
MCT = monocrotaline
PA = pulmonary artery
PAH = pulmonary arterial hypertension
PASMC = pulmonary artery smooth muscle cell
P-EC = pulmonary endothelial cell
PH = pulmonary hypertension
SPH = secondary pulmonary hypertension

METHODS

STUDY POPULATION. Lung specimens were obtained during lung transplantation in 6 patients with idiopathic PAH (IPAH), 6 with secondary pulmonary hypertension (SPH) (3 with sarcoidosis and 3 with pulmonary histiocytosis), and during lobectomy or pneumonectomy for localized lung cancer without PH in 6 control subjects. Control samples were either adenocarcinoma or squamous cell carcinoma. The sampling was always performed on the opposite site of the carcinoma, and lobes with massive emphysema and bronchial inflammation were excluded. Age was 41 ± 10 years and 40 ± 9 years in the patients with IPAH and SPH and 54 ± 12 years in the control subjects. In the groups with IPAH and SPH, the mean pulmonary artery pressure was, respectively, 67 ± 13 mm Hg and 41 ± 16 mm Hg; mean pulmonary vascular resistance was 25.2 ± 4 and 17 ± 9 mm $Hg \cdot l^{-1} \cdot min \cdot m^2$ and mean cardiac index was 2.1 ± 0.3 and 4 ± 2 $l \cdot min^{-1} \cdot m^{-2}$. Transthoracic echocardiography was performed preoperatively in the control subjects to rule out PH. Patients with a mutation in the bone morphogenic protein receptor II (BMPRII) gene were excluded from this study. Study patients were part of the French Network on Pulmonary Hypertension, a program approved by our institutional Ethics Committee, and had given written informed consent (Protocol N8CO-08-003, ID RCB: 2008-A00485-50, approved on June 18, 2008).

ISOLATION AND CULTURE OF HUMAN P-ECs AND PASMCs. Human P-ECs and PASMCs were cultured as previously described (11) and were used for the study between passages 3 and 5.

PREPARATION OF HUMAN P-EC–CONDITIONED MEDIUM. P-ECs from control subjects and from IPAH patients were seeded in 6-well plates at a density of

25×10^4 cells per well and allowed to adhere and grow in MCDB131 medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS), 50 U/mL of penicillin/streptomycin, 4 mmol/l L-glutamine, 25 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 U/ml heparin, 1 μg/ml human endothelial cell growth supplement, and 10 ng/ml vascular endothelial growth factor (Promocell, Heidelberg, Germany) for 24 h. The P-ECs were then serum-starved in MCDB131 medium with or without increasing concentrations of nebivolol or metoprolol (10^{-6} to 10^{-4} mol/l). After incubation for 24 h, the medium was collected for PA-SMC growth assay and for enzyme-linked immunoassays (all from R&D Systems, Lille, France).

MEASUREMENTS OF P-EC PROLIFERATION. P-ECs were seeded in 96-well plates at a density of 5×10^3 cells per well and allowed to adhere in the MCDB-131 supplemented with 15% FCS for 24 h. The P-ECs were then serum-starved in MCDB131 medium for the next 24 h. The cells were subjected to 24 h in medium containing 0% or 15% FCS in the presence of increasing concentrations of nebivolol or metoprolol (10^{-6} mol/l to 10^{-4} mol/l). During this period, the DNA synthesis in proliferating cells was measured by use of the DELPHIA Cell Proliferation Kits (Perkin Elmer, Courtaboeuf, France). Each measurement was performed in triplicate.

MEASUREMENTS OF PASMC PROLIFERATION. Control PASMCs in Dulbecco's modified Eagle's medium supplemented with 15% FCS were seeded in 96-well plates at a density of 5×10^3 cells per well and allowed to adhere. The cells were subjected to 48 h of growth arrest in medium containing 0% FCS and then treated with 200 μl P-EC-conditioned medium for 24 h. Cell growth was then measured by use of the DELPHIA Cell Proliferation Kits (Perkin Elmer). Each measurement was performed in triplicate.

IN VIVO STUDY DESIGN, MEASUREMENTS, AND TISSUE SAMPLING. Experiments were conducted according to the European Union regulations (Directive 86/609 EEC) for animal experiments and complied with our institution's guidelines for animal care and handling. The animal facility is licensed by the French Ministry of Agriculture (agreement No. B92-019-01). The Committee on the Ethics of Animal Experiments CEEA26 CAPSud approved the study. Dr. Perros supervised all animal experiments (agreement delivered by the French Ministry of Agriculture for animal experiment No. A92-392). All efforts were made to minimize animal suffering.

It has been shown that responses to PH triggers are significantly affected by age, because younger individuals with rapidly maturing lungs are more susceptible to these triggers (12,13). In this study, we used very young non-adult rats at 100 g weight exposed to MCT to get a model of very severe PH with right heart failure.

Male Wistar rats (100 g body weight) were maintained in a temperature-controlled room with a 12/12-h light/dark cycle and randomly divided into: 1) a saline-treated control group (control, n = 10); 2) a monocrotaline-exposed group (MCT, n = 10); 3) an MCT-exposed and 10 $mg \cdot kg^{-1} \cdot day^{-1}$ (days 14-21) nebivolol-treated group (MCT+N10, n = 10); 4) an MCT-exposed and 10 $mg/kg^1/day^1$ (days 14-21) metoprolol-treated group (MCT+M10, n = 10); and 5) an MCT-exposed and 100 $mg \cdot kg^{-1} \cdot day^{-1}$ (days 14-21) metoprolol-treated group (MCT+M100, n = 10). All rats had access to standard rat chow and water ad libitum. For MCT administration, rats received a single subcutaneous injection of 60 $mg \cdot kg^{-1}$ MCT (Sigma-Aldrich, Lyon, France), which was dissolved in 1 N HCl and neutralized with 1 N NaOH. At day 21, measurement of the right ventricular systolic pressure (RVSP) (mm Hg), mean pulmonary artery pressure (mPAP) (mm Hg), cardiac output (CO) $ml \cdot min^{-1}$, and total pulmonary vascular resistances (PVR) $mm\ Hg \cdot ml \cdot min^{-1}$ were recorded as previously described (14). After rats were exsanguinated, the right lungs were distended by infusion of optimal cutting temperature compound (Miles, Epernon, France) diluted in phosphate-buffered saline (1:1) into the right principal bronchus, quick-frozen in isopentane on dry ice, and stored at -80°C. Left lungs were formalin-distended, fixed, and paraffin-embedded. For Fulton's index of right ventricular hypertrophy, the ratio of the right ventricular weight-to-left ventricular plus septal weight (RV/LV+S) was calculated. In a second experiment, we treated MCT-exposed rats with nebivolol during 14 days (days 14-28, MCT+N10, n = 9), as compared with MCT alone (n = 15) and control (n = 5). In this second experiment, systolic carotid artery pressure and heart rate were also analyzed for the possible systemic side effects of nebivolol treatment.

PULMONARY ARTERY MORPHOMETRY AND QUANTIFICATION OF PULMONARY INFLAMMATION. After paraffin embedding, 5-μm-thick lung sections were stained with hematoxylin-phloxine-saffron. In each rat, 40 to 60 intra-acinar arteries were analyzed and categorized as muscularized (fully or partially) or nonmuscularized to assess the degree of muscularization.

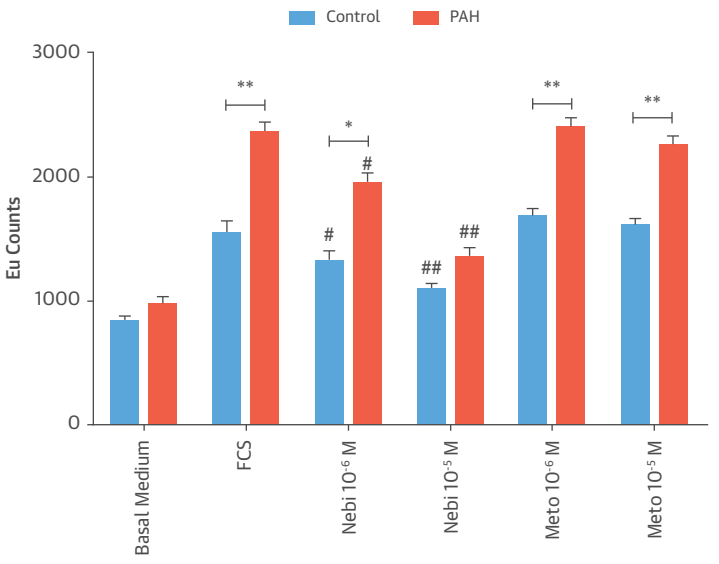
Pulmonary macrophages were stained on 6-μm sections of frozen tissue with a mouse anti-rat CD68

(clone ED1, Serotec, Oxford, United Kingdom) and detected with a donkey anti-mouse alexa-fluor 594. Cell quantification was performed in 4 wide fields by use of an automated counting with a Nikon eclipse 80i camera (Nikon France SAS, Champigny sur Marne, France) and NIH image software (freeware, available from: http://rsb.info.nih.gov/nih-image/Default.html). Results are expressed as number of cells by field.

REAL-TIME POLYMERASE CHAIN REACTION. We performed real-time polymerase chain reaction as previously described (15), with the use of TaqMan gene expression assays (ID number in brackets) (Gapgh [Rn01775763_g1] and Bnp [Rn00676450_g1]) on a StepOnePlus system (Applied Biosystems, Courtaboeuf, France).

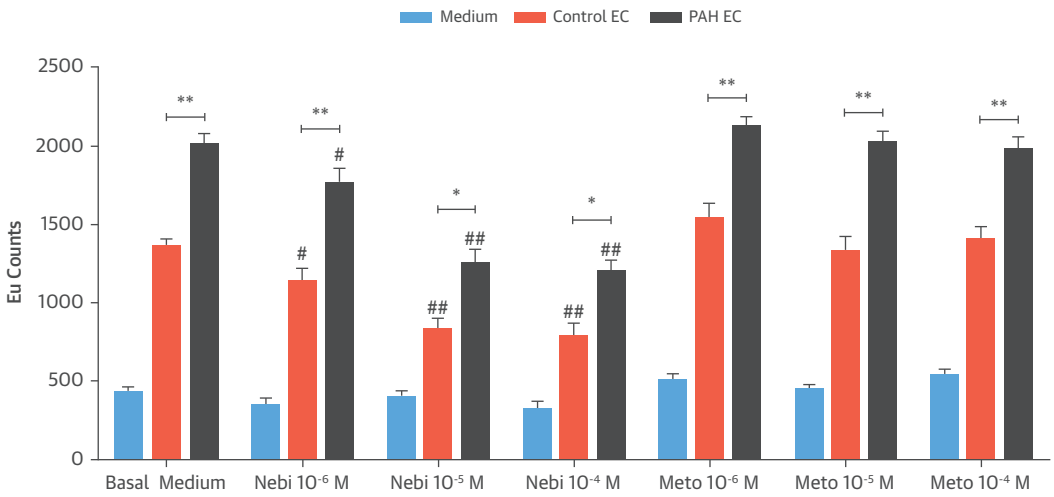
ORGAN BATH STUDIES. Male Wistar rats were killed by intraperitoneal overdose of sodium pentobarbital. The lung was removed and the pulmonary arteries were gently dissected of adjacent tissue. Ring segments (average diameter = 1 mm and length = 2 mm) were cleaned and cut gently; care was taken to preserve the endothelium. In some experiments, the endothelium was deliberately removed by rubbing the lumen. The arteries were then suspended in glass organ baths containing oxygenated Krebs solution. Resting tension of 0.2 g force was maintained

FIGURE 1 In Vitro Effects of Nebivolol and Metoprolol on Human Pulmonary Endothelial Cell Proliferation



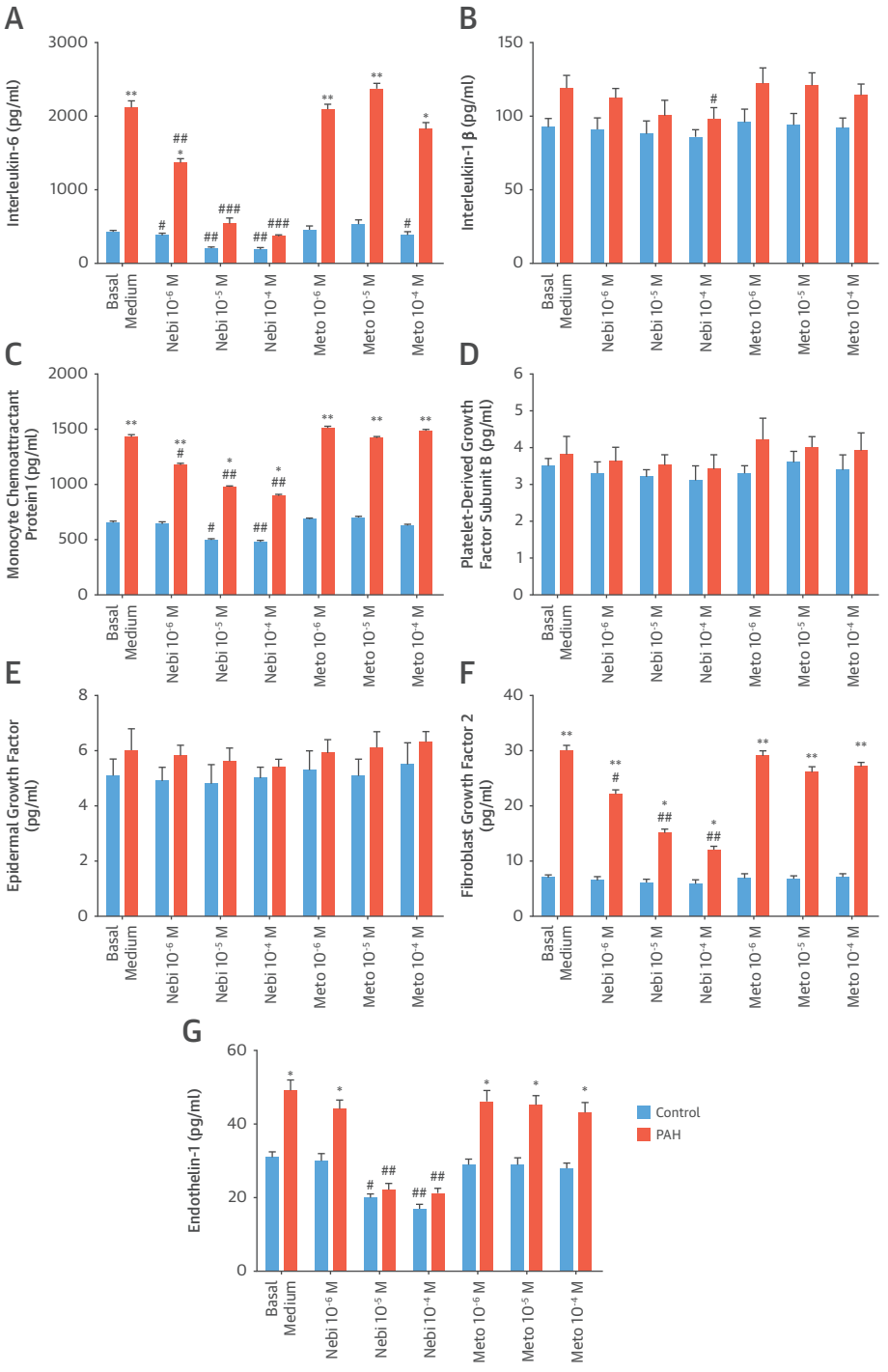
Pulmonary endothelial cells (P-EC) from control and pulmonary arterial hypertension (PAH) lungs were either cultured with basal medium, fetal calf serum (FCS), or FCS + nebivolol (Nebi) or FCS + metoprolol (Meto) at 10^{-6} mol/l and 10^{-5} mol/l. Results are expressed in europium counts (Eu count). Comparison between control and PAH: *p < 0.05, **p < 0.01. Comparison to cells in the same group without treatment: #p < 0.05, ##p < 0.01. n = 6 in each condition.

FIGURE 2 In Vitro Effects of Nebivolol and Metoprolol on Human Pulmonary Artery Smooth Muscle Cell Proliferation Induced by Pulmonary Endothelial Cells (Pulmonary Endothelial Cell to Pulmonary Artery Smooth Muscle Cell Crosstalk)



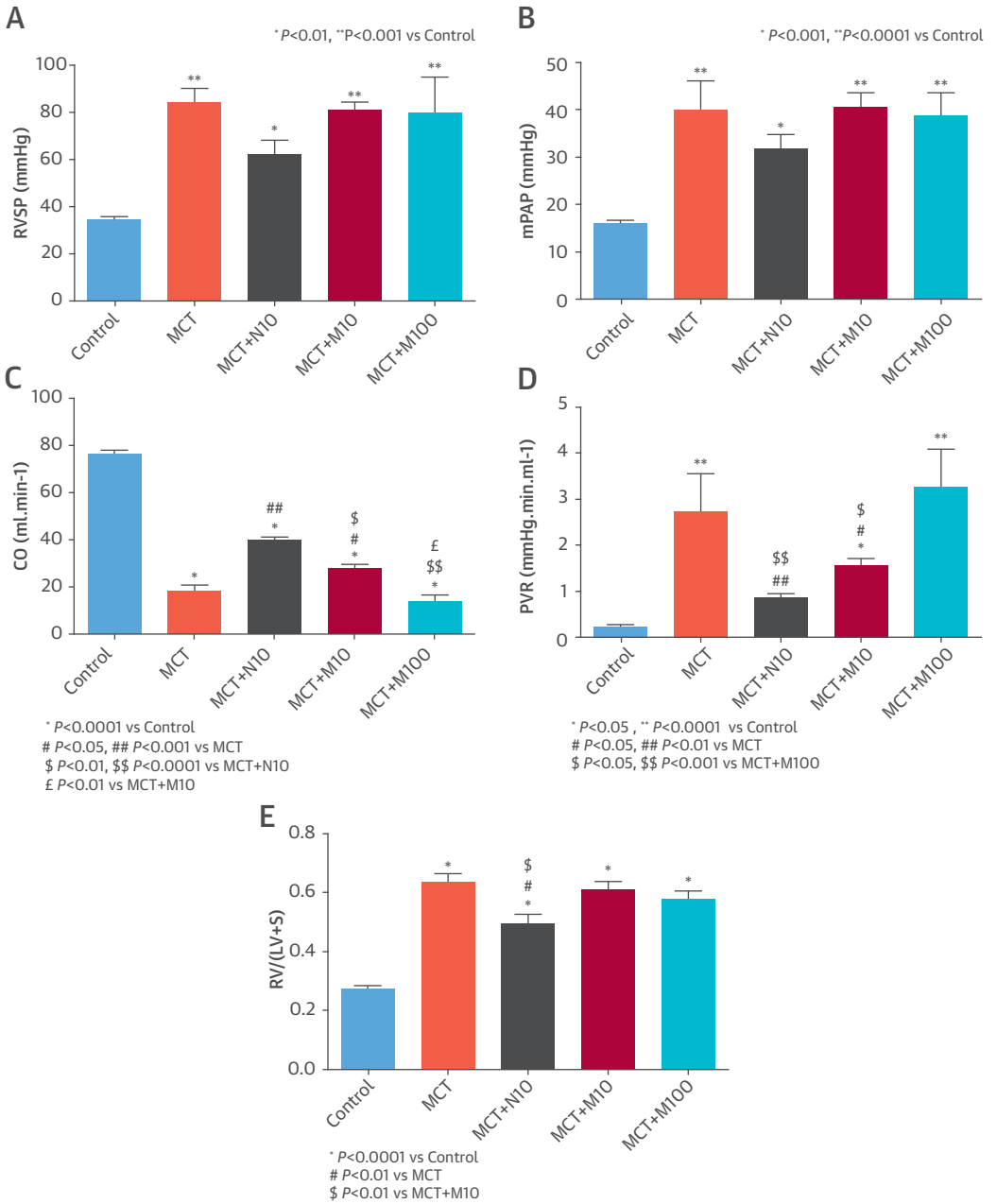
Pulmonary artery smooth muscle cells (PASMC) were either cultured with basal medium or human pulmonary endothelial cell (P-EC)-conditioned medium from control and PAH patients, with or without nebivolol (Nebi) or metoprolol (Meto) at 10^{-6} mol/l, 10^{-5} mol/l, and 10^{-4} mol/l. Results are expressed in europium counts (Eu count). Comparison between control and PAH: *p < 0.05, **p < 0.01. Comparison to cells in the same group without treatment: #p < 0.01, ##p < 0.001. n = 6 in each condition.

FIGURE 3 **In Vitro Effects of Nebivolol and Metoprolol on Human Pulmonary Endothelial Cell Production of Proinflammatory Cytokines/Chemokines (IL-6, IL-1β, and MCP-1), Growth Factors (PDGF, EGF, and FGF-2), and Vasoconstrictive Factor (ET-1)**



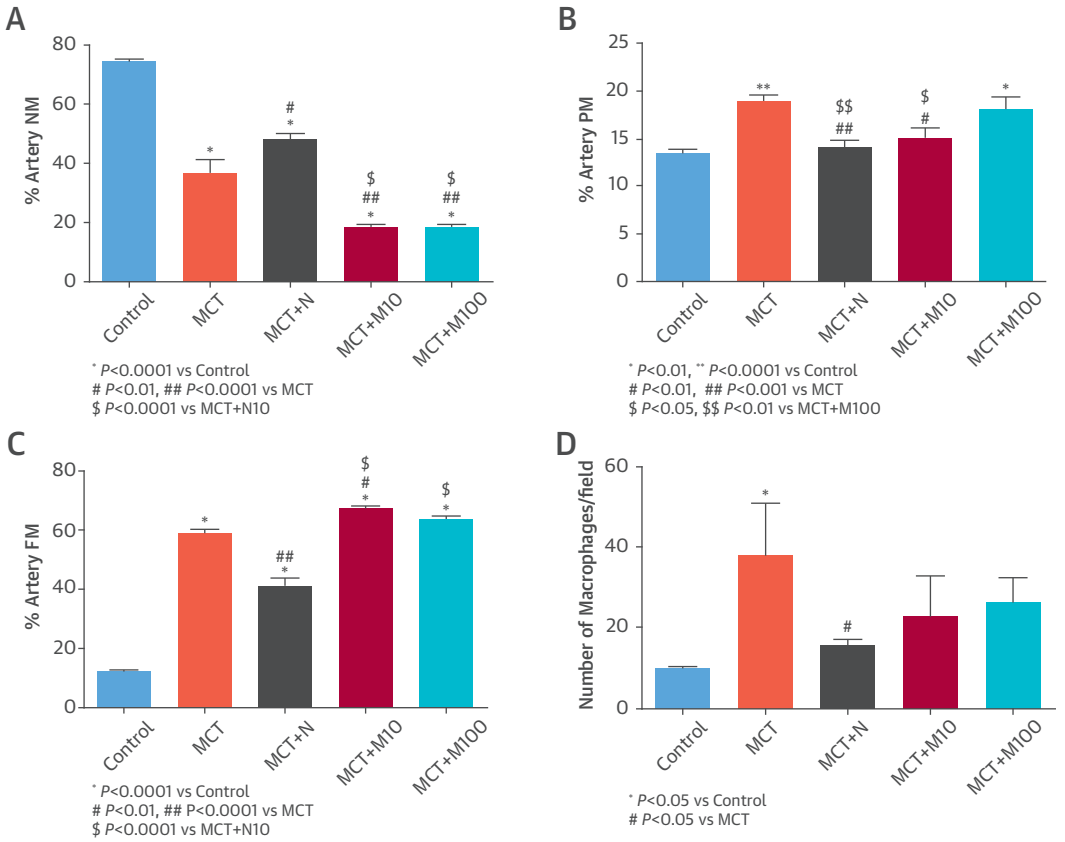
The pulmonary endothelial cells (P-ECs) from control and pulmonary arterial hypertension (PAH) lungs were serum-starved in MCDB131 medium with or without increasing concentrations of nebivolol or metoprolol (10^{-6} to 10^{-4} mol/l). After incubation for 24 h, the medium was collected for enzyme-linked immunoassay. Comparison between control and PAH: *p < 0.01, **p < 0.001. **(A)** interleukin-6, **(B)** interleukin-1β, **(C)** monocyte chemoattractant protein 1, **(D)** platelet-derived growth factor subunit B, **(E)** epidermal growth factor, **(F)** fibroblast growth factor 2, and **(G)** endothelin-1. Comparison to cells in the same group without treatment: #p < 0.05, ##p < 0.01, ###p < 0.001. n = 6 in each condition.

FIGURE 4 **In Vivo Effects of Nebivolol and Metoprolol on Hemodynamics and Right Heart Hypertrophy in Monocrotaline-Induced Pulmonary Hypertension in Rats**



Five groups were compared: 1) saline-treated control group (control); 2) monocrotaline (MCT)-exposed group; 3) MCT-exposed and 10 mg·kg^{-1}·day^{-1} (days 14–21) nebivolol-treated group (MCT + N10); 4) MCT-exposed and 10 mg·kg^{-1}·day^{-1} (days 14–21) metoprolol-treated group (MCT + M10); and 5) MCT-exposed and 100 mg·kg^{-1}·day^{-1} (days 14–21) metoprolol-treated group (MCT + M100). In vivo effects of nebivolol and metoprolol on **(A)** right ventricular systolic pressures (RVSP) (mm Hg), **(B)** mean pulmonary artery pressure (mPAP) (mm Hg), **(C)** cardiac output (CO) (ml·min^{-1}), **(D)** total pulmonary vascular resistance (PVR) (mm Hg·min·ml^{-1}), and **(E)** Fulton's index of right ventricular hypertrophy, calculated as the ratio of the right ventricular weight-to-left ventricular plus septal weight (RV/LV+S). Control, n = 10; MCT, n = 5; MCT + N10, n = 7; MCT + M10, n = 6; MCT + M100, n = 4.

FIGURE 5 In Vivo Effects of Nebivolol and Metoprolol on Pulmonary Vascular Remodeling and Inflammation in Monocrotaline-Induced Pulmonary Hypertension in Rats



Five groups were compared: 1) saline-treated control group (control); 2) monocrotaline MCT-exposed group; 3) MCT-exposed and 10 $mg \cdot kg^{-1} \cdot day^{-1}$ (days 14–21) nebivolol-treated group (MCT + N10); 4) MCT-exposed and 10 $mg \cdot kg^{-1} \cdot day^{-1}$ (days 14–21) metoprolol-treated group (MCT + M10); and 5) MCT-exposed and 100 $mg \cdot kg^{-1} \cdot day^{-1}$ (days 14–21) metoprolol-treated group (MCT + M100). The analysis of the neomuscularization of normally non-muscularized small distal pulmonary artery (PA) (≤50 μm) is a common and robust way to quantify the degree of remodeling of the rat pulmonary vasculature. **(A)** Percentage of non-muscularized (NM) PA. **(B)** Percentage of partially muscularized (PM) PA. **(C)** Percentage of fully muscularized (FM) PA. **(D)** Mean number of macrophages (CD68+ cells) per low-magnification field. **(E)** Representative images of pulmonary vascular remodeling and macrophage accumulation in the 5 different groups (control, MCT + N, MCT + M10, and MCT + M100). Control, n = 10; MCT, n = 5; MCT + N10, n = 7; MCT + M10, n = 6; MCT + M100, n = 4.

Continued on the next page

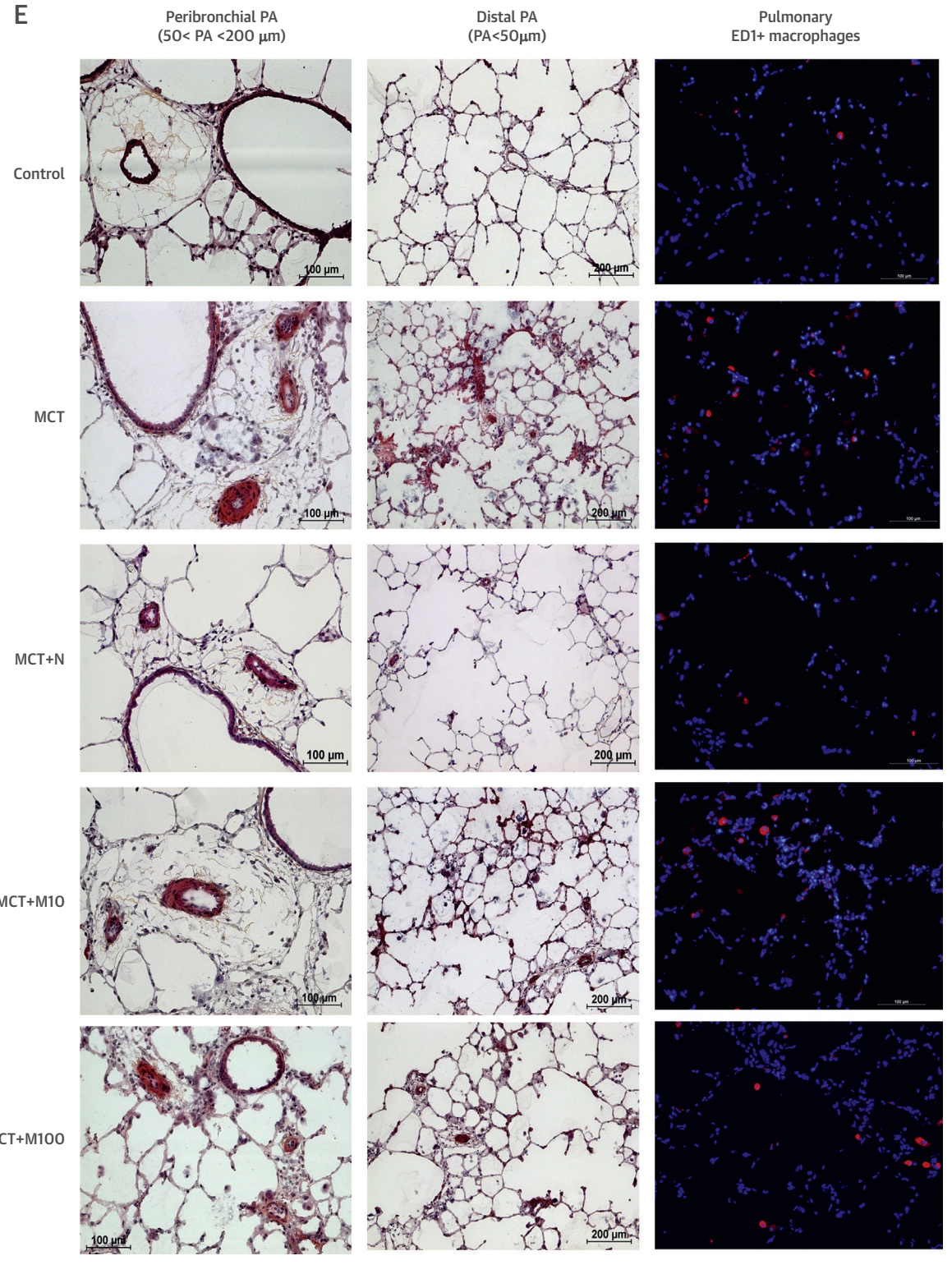
throughout the experiment. Tissues were allowed to equilibrate for 2 h before the addition of any drugs. Contraction was elicited with the use of 0.1 μmol/l norepinephrine; at the maximal response, we added acetylcholine (10^{-8} to 10^{-5} mol/l) to test the endothelium integrity. The tissues were then washed with fresh Krebs-Ringer solution after 60 min of re-equilibration; subsequent contractile response to norepinephrine was performed, and nebivolol or metoprolol was added at increasing concentrations (10^{-8} to 10^{-4} mol/l). The same experiments were performed on arteries pre-treated with the NO synthase (NOS) inhibitor NG-nitro-L-arginine methyl ester (L-NAME) (10^{-4} mol/l).

STATISTICAL EVALUATION. All data were verified for normal distribution. Quantitative variables are presented as mean ± SEM. Data were analyzed with use of the Student *t* test or one-way analysis of variance and Bonferroni multiple comparisons test. Values of $p < 0.05$ were considered to reflect statistical significance.

RESULTS

NEBIVOLOL BUT NOT METOPROLOL REDUCES HUMAN P-ECs AND P-EC–INDUCED PASMC PROLIFERATION. As expected (16), human P-ECs from IPAH lungs proliferated more than did control P-ECs under basal

FIGURE 5 Continued

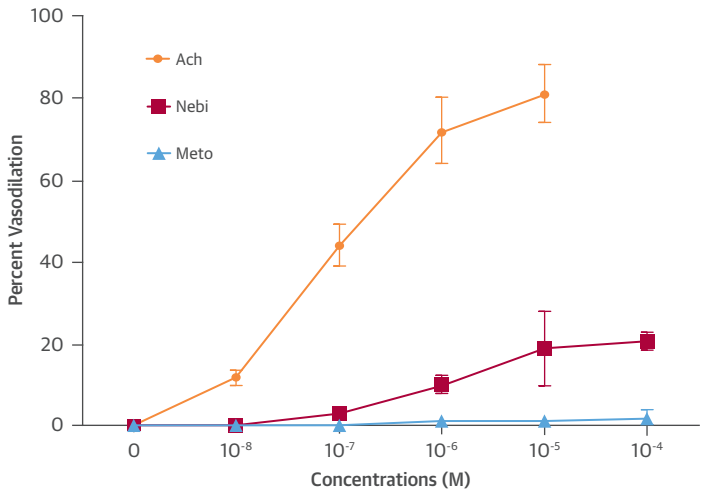


and FCS-stimulated conditions (Figure 1). Nebivolol significantly decreased FCS-induced P-EC proliferation in a dose-dependent fashion by a 1.8 reduction factor in IPAH and by 1.4 in control cells (both $p < 0.01$) at 10 μmol/l. Metoprolol had no significant effect on FCS-stimulated proliferation of IPAH and control P-ECs. We tested the effects of nebivolol and metoprolol on P-EC–induced PASMC proliferation by transferring P-EC medium conditioned with or without β-blockers to PASMC cultures. As already shown (17), P-EC–conditioned medium from IPAH cells induced significantly more PASMC proliferation as compared with control ($p < 0.01$) (Figure 2). Nebivolol but not metoprolol decreased this mitogenic crosstalk in a dose-dependent fashion of both IPAH and control P-EC medium (both $p < 0.001$ at 10 μmol/l nebivolol). Of note, 100 μmol/l nebivolol did not reach a significantly higher blocking effect as compared with 10 μmol/l. Nebivolol had similar effects on P-ECs from SPH lungs (Online Figures 1 and 2).

NEBIVOLOL BUT NOT METOPROLOL REDUCES THE PATHOLOGICAL OVERPRODUCTION OF INTERLEUKIN-6, MONOCYTE CHEMOATTRACTANT PROTEIN-1, FIBROBLAST GROWTH FACTOR-2, AND ET-1 FOUND IN HUMAN IPAH P-ECs. With the use of an enzyme-linked immunoassay, we quantified the proinflammatory cytokines/chemokine (interleukin [IL]1-β, IL-6, and monocyte chemoattractant protein 1 [MCP1]), the growth factors (PDGF, EGF, and FGF2), and the vasoconstrictive factor ET-1 concentrations in P-EC–conditioned mediums. We found an overexpression of IL-6, MCP-1, FGF2, and ET-1 in IPAH cells (Figures 3A, 3C, 3F, and 3G) ($p < 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively, in IPAH compared with control P-EC). However, we did not detect changes in IL1-β, PDGF, and EGF production (Figures 3B, 3D, and 3E) in IPAH compared with control P-EC–conditioned mediums. These alterations were either reduced (MCP-1 and FGF2) or completely normalized (IL-6 and ET-1) by nebivolol but not by metoprolol in a dose-dependent fashion. Nebivolol had similar effects on P-EC from SPH lungs (Online Figure 3).

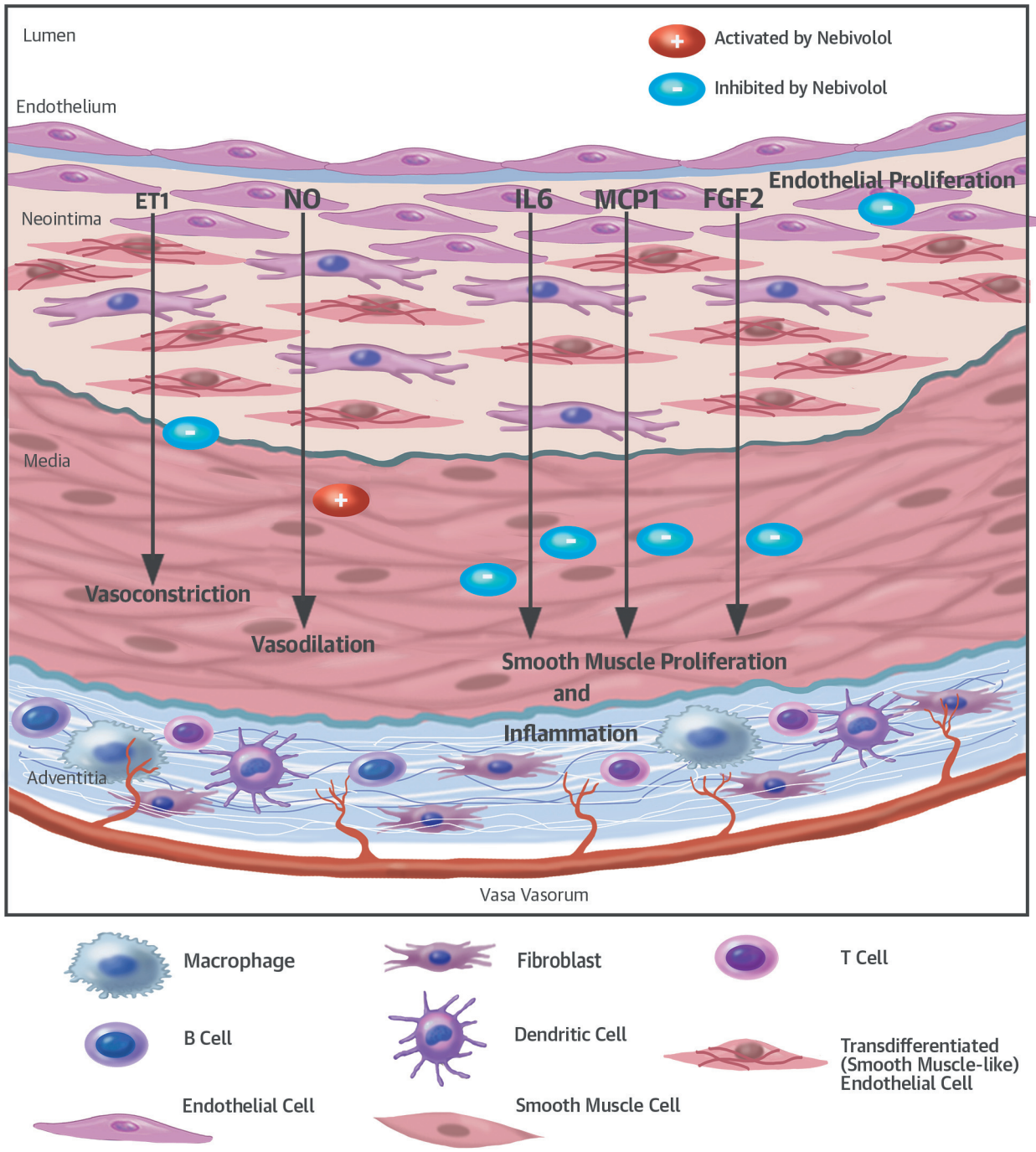
NEBIVOLOL HAS GREATER BENEFICIAL EFFECTS THAN METOPROLOL ON EXPERIMENTAL SEVERE PAH. After exposure to MCT (60 mg/kg), young Wistar rats (weight, 100 g) had development of severe PAH. At day 21, MCT-exposed rats had severe PAH characterized by a high RVSP and high mPAP. None of the nebivolol or metoprolol treatments were associated with significant changes in these hemodynamic parameters (Figures 4A and 4B). However, there were significant improvements in CO and PVR in the nebivolol-treated and in the metoprolol-treated (10 mg/kg) groups as compared with non-treated PAH rats ($p < 0.001$ and $p < 0.05$, respectively, as compared with MCT alone-exposed group). Moreover, nebivolol (10 mg/kg) had a better effect on CO than did metoprolol (10 mg/kg) ($p < 0.01$). Metoprolol-treated (100 mg/kg) rats had worse CO and PVR values than did the nebivolol-treated and the metoprolol-treated (10 mg/kg) PAH rats ($p < 0.0001$ and $p < 0.01$, respectively) (Figures 4C and 4D). As the result of the high PVRs that developed in the MCT-exposed rats, there was significant right ventricular hypertrophy assessed by means of the Fulton index (FI = RV/[LV+S]), in these animals ($p < 0.0001$). In accordance with the lower PVRs measured in the nebivolol-treated PAH rats, this group had less RV hypertrophy ($p < 0.01$ as compared with MCT rats). Metoprolol treatment, regardless of dose, did not result in a reduction of RV hypertrophy. There was significantly more RV hypertrophy in the metoprolol-treated (10 mg/kg) group than in the nebivolol-treated group ($p < 0.01$) (Figure 4E). The increase in the PVRs, heart failure, and death in PAH was reflected by progressive remodeling of pulmonary precapillary arteries. The analysis of the neomuscularization of normally non-muscularized

FIGURE 6 Ex Vivo Effects of Nebivolol and Metoprolol on Norepinephrine-Pre-Contracted, Rat Pulmonary Artery Rings



Contraction was elicited with 0.1 μmol/l norepinephrine. At the maximal response, acetylcholine (Ach) (10^{-8} to 10^{-5} mol/l) was used to test the endothelium integrity. The tissues were then washed, subsequent contractile responses to norepinephrine was performed, and nebivolol or metoprolol was added at increasing concentrations (10^{-8} to 10^{-4} mol/l). Drug-induced vasodilation is expressed as percent relaxation from preconstricted diameter. n = 6 rats.

CENTRAL ILLUSTRATION **Pulmonary Hypertension Pathobiology and its Targeting by Nebivolol**



Perros, F. et al. J Am Coll Cardiol. 2015; 65(7):668–80.

Pulmonary artery (PA) endothelial cells from PAH patients (PAH PA-EC) overexpress the proinflammatory mediators interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1, fibroblast growth factor (FGF)-2, and the potent vasoconstrictive agent endothelin (ET)-1. This pathological phenotype is corrected by nebivolol. PAH PA-EC proliferate more than do control cells and stimulate more PA smooth muscle cell mitosis, a growth abnormality that is also normalized by nebivolol. Moreover, nebivolol induced an endothelium-dependent and nitric oxide (NO)-dependent partial relaxation of PA. All taken together, nebivolol reduces pulmonary vascular remodeling and inflammation.

(NM) small distal PA (≤50 μm) is a common and robust way to quantify the degree of remodeling of the rat pulmonary vasculature. We found a dramatic decrease in the low-resistance NM PA in MCT-exposed rats as compared with control rats ($p < 0.0001$) and its corollary, a dramatic increase in high-resistance, fully muscularized (FM) PA ($p < 0.0001$) (**Figures 5A and 5C**). In addition, there was an increase in partially muscularized (PM) PA in MCT-exposed rats as compared with control ($p < 0.0001$) (**Figure 5B**). Nebivolol (10 mg/kg) increased the percentage of NM PA ($p < 0.01$ as compared with MCT alone–exposed group), whereas metoprolol (both doses) further decreased this percentage, as compared with MCT-exposed rats (both $p < 0.0001$) (**Figure 5A**). In contrast, nebivolol decreased the percentage of PM and FM PA (both $p < 0.01$), whereas metoprolol (10 mg/kg) decreased the percentage of PM PA ($p < 0.01$) but increased further the percentage of FM PA ($p < 0.01$) as compared with MCT-exposed rats. Metoprolol (100 mg/kg) had higher percentages of both PM and FM as compared with nebivolol-treated rats ($p < 0.01$ and $p < 0.0001$, respectively) (**Figures 5A and 5C**). At last, nebivolol decreased significantly the accumulation of macrophages in the MCT lung ($p < 0.05$) (**Figure 5D**). Representative images of the pulmonary vascular remodeling and macrophage accumulation in the 5 different groups (control, MCT, MCT+N, MCT+M10, and MCT+M100) are shown in **Figure 5E**. By treating MCT-exposed rats for a longer duration (14 days, from day 14 to day 28 after MCT), we observed more beneficial effects of nebivolol on RVSP, mPAP, and RV hypertrophy, associated with a decrease in the right ventricular BNP expression. CO was not improved, but PVR was reduced (Online Figure 4). However, it is difficult to compare the effects of nebivolol at day 21 (considered as early and compensated PH) and at day 28 (late and decompensated PH). The effect on the distal muscularization was similar as observed at day 28 as compared with day 21 (Online Figure 5).

NEBIVOLOL BUT NOT METOPROLOL INDUCED VASODILATION OF PRECONTRACTED RAT PA RINGS. Nebivolol allowed a vasodilation of ≈20% on norepinephrine-induced, precontracted rat PA rings at 10^{-5} and 10^{-4} mol/l, whereas metoprolol had no effect (**Figure 6**). When the endothelium was removed or when the NOS was inhibited by L-NAME, Ach- and nebivolol-induced PA vasodilation were totally abolished (data not shown).

DISCUSSION

We found that PAH P-ECs overexpressed the proinflammatory mediators IL-6 and MCP-1, the growth factor FGF2, and the potent vasoconstrictive agent endothelin-1 as compared with control cells. This pathological phenotype was corrected by the use of nebivolol but not metoprolol in a dose-dependent fashion. We confirmed that PAH P-ECs proliferate more than control cells and stimulate more PASMC mitosis, a growth abnormality that was normalized by nebivolol but not by metoprolol. Interestingly, nebivolol had similar effects on P-ECs from SPH lungs. Nebivolol induced an endothelium- and NO-dependent partial relaxation of PA. At last, nebivolol was more potent than was metoprolol in improving cardiac function, pulmonary vascular remodeling, and inflammation of rats with MCT-induced PH.

Nebivolol is a third-generation β-adrenergic receptor blocker with vasodilator properties mediated by stimulation of endothelial NO synthase (eNOS) (18). Accordingly, we found that L-NAME totally abolished the vasodilatory properties of nebivolol on isolated PA in organ bath experiments. In our study, nebivolol was more potent than metoprolol to correct PAH-related endothelial dysfunction in vitro and to improve experimental PAH in rats. These results are consistent with previous work showing that nebivolol but not metoprolol normalizes endothelial function, reduces superoxide formation, increases NO bioavailability, and inhibits up-regulation of the activity and expression of the vascular NAD(P)H oxidase in a model of angiotensin II-induced oxidative stress (19), thus preventing eNOS uncoupling. Nebivolol also reduces the levels of the circulating eNOS inhibitor asymmetric dimethylarginine, which probably contributes to increased vascular NO bioavailability, whereas metoprolol does not contribute (20). We also observed that nebivolol corrected better than did metoprolol the proinflammatory phenotype of the P-ECs from PAH patients (reduction of IL-6 and MCP1 expression) and dramatically decreased macrophage accumulation in the MCT lung. In vivo, the anti-inflammatory properties of nebivolol, and, to a lesser extent, metoprolol, may also be due to a direct effect on immune cells, because catecholamines are actively produced by macrophages and have the capacity to act in an autocrine way on ARs to regulate macrophage production of IL-1β, which has a key role in the inflammatory response (21). In our study, nebivolol treatment

decreased vascular remodeling in experimental PH, whereas metoprolol worsened it. One reason for this divergence, apart from all beneficial effects of nebivolol that are not shared with metoprolol, may be an antagonist effect of metoprolol on vascular β_2-ARs even if metoprolol has a good β_1 selectivity (β_1/β_2 selectivity = 74) (18). This also may be true at a higher dose of metoprolol (100 mg/kg). High concentrations of metoprolol may favor PA constriction and PASMC proliferation. MCT exposure is a model of severe PH, in which all rats die of right heart failure, and both β-blockers may have improved right heart function/coupling. Nebivolol has been shown to preserve left ventricular function, cause peripheral vasodilation, maintain stroke volume and cardiac output, and preserve cardiac chronotropism during exertion (18). Furthermore, compared with bisoprolol, nebivolol did not cause an increase in pulmonary artery wedge pressure (9). This could further explain the superiority of nebivolol over metoprolol to improve experimental PH. However, one must be aware that the MCT-induced PH is considered by some to be an acute toxic model characterized by acute/subacute damage of the peripheral vasculature of the lung and other organs (kidney, liver, and heart) (13). For this reason, we plan to test the efficacy of nebivolol in a piglet model of chronic PH, which is more relevant to the human condition (22–24).

CONCLUSIONS

Our work demonstrates for the first time that nebivolol could be a promising option for the management of PAH, improving endothelial dysfunction, pulmonary vascular remodeling, and right heart function. Until clinical studies are undertaken, however, routine use of β-blockers in PAH cannot be recommended.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The third-generation β-adrenergic receptor blocker nebivolol appears to attenuate experimental pulmonary hypertension by correcting endothelial dysfunction (**Central Illustration**).

TRANSLATIONAL OUTLOOK: Although β-adrenergic receptor blockade is not currently recommended for treatment of patients with pulmonary arterial hypertension, clinical trials of nebivolol are warranted on the basis of this distinguishing mechanism of action.

REFERENCES

1. Humbert M, Montani D, Perros F, et al. Endothelial cell dysfunction and cross talk between endothelium and smooth muscle cells in pulmonary arterial hypertension. Vascul Pharmacol 2008;49:113–8.

2. Humbert M, Sitbon O, Simonneau G. Treatment of pulmonary arterial hypertension. N Engl J Med 2004;351:1425–36.

3. Humbert M, Sitbon O, Chaouat A, et al. Survival in patients with idiopathic, familial, and anorexigen-associated pulmonary arterial hypertension in the modern management era. Circulation 2010;122:156–63.

4. Hoeper MM, Barst RJ, Bourge RC, et al. Imatinib mesylate as add-on therapy for pulmonary arterial hypertension: results of the randomized IMPRES study. Circulation 2013;127:1128–38.

5. Bogaard HJ, Natarajan R, Mizuno S, et al. Adrenergic receptor blockade reverses right heart remodeling and dysfunction in pulmonary hypertensive rats. Am J Respir Crit Care Med 2010;182:652–60.

6. De Man FS, Handoko ML, van Ballegoij JJM, et al. Bisoprolol delays progression towards right heart failure in experimental pulmonary hypertension. Circ Heart Fail 2012;5:97–105.

7. De Man FS, Handoko ML, Guignabert C, et al. Neurohormonal axis in patients with pulmonary arterial hypertension: friend or foe? Am J Respir Crit Care Med 2013;187:14–9.

8. Drake JI, Gomez-Arroyo J, Dumur CI, et al. Chronic carvedilol treatment partially reverses the right ventricular failure transcriptional profile in experimental pulmonary hypertension. Physio Genomics 2013;45:449–61.

9. Kamp O, Metra M, Bugatti S, et al. Nebivolol: haemodynamic effects and clinical significance of combined beta-blockade and nitric oxide release. Drugs 2010;70:41–56.

10. Martiniuc C, Branishte T. The use of beta blocker Nebivolol in patients with chronic obstructive pulmonary disease in association with arterial hypertension. Rev Med Chir Soc Med Nat Iasi 2012;116:218–21.

11. Eddahibi S, Guignabert C, Barlier-Mur A-M, et al. Cross talk between endothelial and smooth muscle cells in pulmonary hypertension: critical role for serotonin-induced smooth muscle hyperplasia. Circulation 2006;113:1857–64.

12. Naeije R, Dewachter L. Animal models of pulmonary arterial hypertension. Rev Mal Respir 2007;24:481–96.

13. Stenmark KR, Meyrick B, Galie N, et al. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. Am J Physiol Lung Cell Mol Physiol 2009;297:L1013–32.

14. Price LC, Montani D, Tcherakian C, et al. Dexamethasone reverses monocrotaline-induced pulmonary arterial hypertension in rats. Eur Respir J 2011;37:813–22.

15. Perros F, Montani D, Dorfmüller P, et al. Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med 2008;178:81–8.

16. Tu L, De Man FS, Girerd B, et al. A critical role for p130Cas in the progression of pulmonary hypertension in humans and rodents. Am J Respir Crit Care M 2012;186:666–76.

17. Dewachter L, Adnot S, Fadel E, et al. Angiopoietin/Tie2 pathway influences smooth muscle

hyperplasia in idiopathic pulmonary hypertension. Am J Respir Crit Care Med 2006;174:1025–33.

18. Münzel T, Gori T. Nebivolol: the somewhat-different beta-adrenergic receptor blocker. J Am Coll Cardiol 2009;54:1491–9.

19. Oelze M, Daiber A, Brandes RP, et al. Nebivolol inhibits superoxide formation by NADPH oxidase and endothelial dysfunction in angiotensin II-treated rats. Hypertension 2006;48:677–84.

20. Kandavar R, Higashi Y, Chen W, et al. The effect of nebivolol versus metoprolol succinate extended release on asymmetric dimethylarginine in hypertension. J Am Soc Hypertens 2011;5:161–5.

21. Engler KL, Rudd ML, Ryan JJ, et al. Autocrine actions of macrophage-derived catecholamines on interleukin-1 beta. J Neuroimmunol 2005;160:87–91.

22. Boulate D, Perros F, Dorfmuller P, et al. Pulmonary microvascular lesions regress in reperfused chronic thromboembolic pulmonary hypertension. J Heart Lung Transplant 2014 Jul 16 [E-pub ahead of print].

23. Guihaire J, Haddad F, Boulate D, et al. Right ventricular plasticity in a porcine model of chronic pressure overload. J Heart Lung Transplant 2014;33:194–202.

24. Mercier O, Tivane A, Dorfmüller P, et al. Piglet model of chronic pulmonary hypertension. Pulm Circ 2013;3:908–15.

KEY WORDS β-blocker, endothelial dysfunction, inflammation, nebivolol, pulmonary hypertension

APPENDIX For supplemental figures, please see the online version of this article.