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Role of Nerve Growth Factor in Development and Persistence of Experimental Pulmonary Hypertension

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Abstract

Rationale: Pulmonary hypertension (PH) is characterized by a progressive elevation in mean pulmonary arterial pressure, often leading to right ventricular failure and death. Growth factors play significant roles in the pathogenesis of PH, and their targeting may therefore offer novel therapeutic strategies in this disease.

Objectives: To evaluate the nerve growth factor (NGF) as a potential new target in PH.

Methods: Expression and/or activation of NGF and its receptors were evaluated in rat experimental PH induced by chronic hypoxia or monocrotaline and in human PH (idiopathic or associated with chronic obstructive pulmonary disease). Effects of exogenous NGF were evaluated *ex vivo* on pulmonary arterial inflammation and contraction, and *in vitro* on pulmonary vascular cell proliferation, migration, and cytokine secretion. Effects of NGF inhibition were

evaluated *in vivo* with anti-NGF blocking antibodies administered both in rat chronic hypoxia- and monocrotaline-induced PH.

Measurements and Main Results: Our results show increased expression of NGF and/or increased expression/activation of its receptors in experimental and human PH. *Ex vivo/in vitro*, we found out that NGF promotes pulmonary vascular cell proliferation and migration, pulmonary arterial hyperreactivity, and secretion of proinflammatory cytokines. *In vivo*, we demonstrated that anti-NGF blocking antibodies prevent and reverse PH in rats through significant reduction of pulmonary arterial inflammation, hyperreactivity, and remodeling.

Conclusions: This study highlights the critical role of NGF in PH. Because of the recent development of anti-NGF blocking antibodies as a possible new pain treatment, such a therapeutic strategy of NGF inhibition may be of interest in PH.

Keywords: pulmonary circulation; animal disease models; blocking antibodies

Pulmonary hypertension (PH) is characterized by a progressive increase in pulmonary vascular resistance, leading to sustained elevated mean pulmonary arterial

pressure (mPAP) over 25 mm Hg at rest and development of right heart failure (1, 2). Because current therapies slow the progression of the disease but still do not

afford a cure, new therapeutic strategies are required (3).

The pathologic key features of PH occur in small pulmonary arteries and include

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At a Glance Commentary

Scientific Knowledge on the

Subject: Pulmonary hypertension (PH) is a rare but life-threatening disease. Because current therapies remain essentially palliative and do not afford a cure, new therapeutic strategies are required. Pathologic key features of PH occur in small pulmonary arteries and include inflammation, altered vasomotricity, and pulmonary arterial wall remodeling. Many growth factors have been implicated in these events. To identify new therapeutic targets in PH, we focused our attention on the nerve growth factor (NGF), an essential growth factor for neurons, which is produced by lung cells and associated with the development of various pathologic conditions affecting the lung. However, its potential role in PH is unknown.

What This Study Adds to the

Field: Our present study shows increased NGF expression and/or increased expression/activation of its receptors in experimental and human PH. Moreover, it highlights critical roles of NGF in the development and the progression of this disease. Using two well-established animal models of PH, we demonstrate that NGF inhibition with anti-NGF blocking antibodies prevents and reverses PH in rats via its effects on inflammation, pulmonary arterial vasoconstriction, and remodeling. Because NGF inhibition by blocking antibodies is already evaluated as a possible new therapeutic strategy in the treatment of pain, our present work demonstrates that such NGF targeting may offer novel therapeutic perspectives in PH.

inflammation, altered vasomotricity, and remodeling with in particular increased smooth muscle and endothelial cell proliferation and migration (4–6). Growth factors play a major role in these cellular events (7). For instance, platelet-derived growth factor (8, 9) or fibroblast growth factor-2 (10, 11) have been implicated in the pathogenesis of experimental and human PH.

In this context, we focused our attention on the nerve growth factor (NGF), which belongs to the neurotrophin family and is an essential growth factor for neuronal development, survival, and differentiation (12). However, NGF also plays physiologic and pathophysiologic roles outside the nervous system (13). NGF and its receptors, both the picomolar high-affinity tropomyosin-related kinase A (TrkA) and the nanomolar low-affinity p75^{NTR} receptors, are expressed in pulmonary arteries (14). In the airways, NGF stimulates proliferation (15) and migration of human airway smooth muscle cells (16) and migration of human lung fibroblasts (17). In addition, NGF is also a proinflammatory mediator in the airways and participates in bronchial hyperresponsiveness (18). Therefore, we hypothesized that NGF may contribute to pulmonary arterial remodeling, inflammation, and altered reactivity in PH. Thus, we evaluated NGF and NGF receptor expression/activation in experimental PH induced in the rat by chronic hypoxia (CH) or monocrotaline (MCT) exposure, as well as in human PH, either idiopathic pulmonary arterial hypertension (IPAH) or associated with chronic obstructive pulmonary disease (PH-COPD). Then we determined whether exogenous NGF would be able to reproduce *ex vivo* or *in vitro* some pathophysiologic aspects of PH. Finally, we conducted experiments in rats to determine whether anti-NGF blocking antibodies administered *in vivo* prevented and/or reversed pulmonary arterial hyperreactivity, remodeling, and inflammation in CH- or MCT-induced PH. Some of the results of these studies have been previously reported in the form of abstracts (19–23).

Methods

Further details and full description of all methods are given in the online supplement.

Human Materials

Control lung tissues were obtained from 10 patients (control patients) undergoing surgery for lung carcinoma. A preoperative echocardiography was performed for each patient to rule out the presence of PH. After lobectomy and transport in sterile physiologic solution, lung samples, distant from the malignant lesion, were dissected by a pathologist. The absence of tumoral infiltration was retrospectively established in all tissue sections by the histopathologic analysis. Lung tissues were also obtained from five patients with PH-COPD and

12 patients with IPAH, all undergoing lung transplantation. PH was confirmed in all patients by right heart catheterization. The study was approved by local ethics committees (Comité de Protection des Personnes, Sud-Ouest et Outre-mer III, Bordeaux, France; and Comité de Protection des Personnes, Ile de France VII, Le Kremlin Bicêtre, France). Informed consent was obtained from each individual patient.

Human Pulmonary Arterial Cell Cultures

Human pulmonary arterial smooth muscle cells (hPASMCs) were obtained as previously described (24). Human pulmonary arterial endothelial cells (hPAECs; Promocell, Heidelberg, Germany) were cultured according to the manufacturer's instructions. The online supplement provides further details.

Animal Experiments

All animal studies were performed according to approved institutional protocols. Adult male Wistar rats (300–350 g; Janvier Labs, Le Genest Saint-Isle, France) were randomly divided into control, control-treated, PH, and PH-treated groups (n = 4–12 rats per group). PH was induced by either exposing rats to CH in a controlled hypobaric chamber (380 mm Hg) for 28 days, or with a single intraperitoneal MCT injection (60 mg/kg; Sigma-Aldrich). In both preventive and curative protocols, control-treated and PH-treated groups received either nonrelevant antibodies (10 µg/kg intraperitoneally, normal rabbit polyclonal IgG; Millipore, Molsheim, France) or rabbit polyclonal anti-NGF blocking antibodies (10 µg/kg intraperitoneally; Millipore). The online supplement provides further details.

Statistical Analysis

All data are reported as mean ± SEM of N independent observations. Two-way analysis of variance was used to compare concentration–response curves. For all other parameters, one-way analysis of variance was used to assess differences among groups, followed by adapted *post hoc* tests (Mann-Whitney test when comparing two groups, Dunn test when comparing more than two groups). All data were analyzed using GraphPad Prism software v6 (GraphPad Software, La Jolla, CA). *P* less than 0.05 was considered significant.

Results

Expression of NGF and/or Expression/Activation of Its Receptors Are Increased in Experimental and Human PH

In pulmonary arteries from patients with PH-COPD, both NGF secretion (Figure 1A) and protein expression (Figure 1B) were significantly increased compared with control patients ($P < 0.05$ for both). Consistent with these findings, immunohistochemistry showed a strong

NGF staining in the endothelial layer of PH-COPD pulmonary arteries (Figure 1C). By contrast, in patients with IPAH, no differences in NGF serum levels or in lung protein expression were observed compared with control subjects (Figures 1D and 1E). In animal models of PH, NGF secretion by pulmonary arteries was significantly increased in both CH-exposed ($P < 0.05$) and MCT-challenged rats ($P < 0.001$) compared with control rats (Figure 1F). NGF protein expression was also significantly increased in pulmonary arteries from

CH-exposed ($P < 0.05$) (Figure 1G) and MCT-challenged rats ($P < 0.01$) (Figure 1H) compared with control animals. Increased expression of NGF was also evidenced by immunohistochemistry in CH-exposed and MCT-challenged rats (Figure 1I).

Protein expression of both TrkA and p75^{NTR} receptors was detected in pulmonary arteries from control patients and from control rats (Figure 2). Expression of the TrkA receptor was modified neither in patients with PH-COPD nor in patients with IPAH

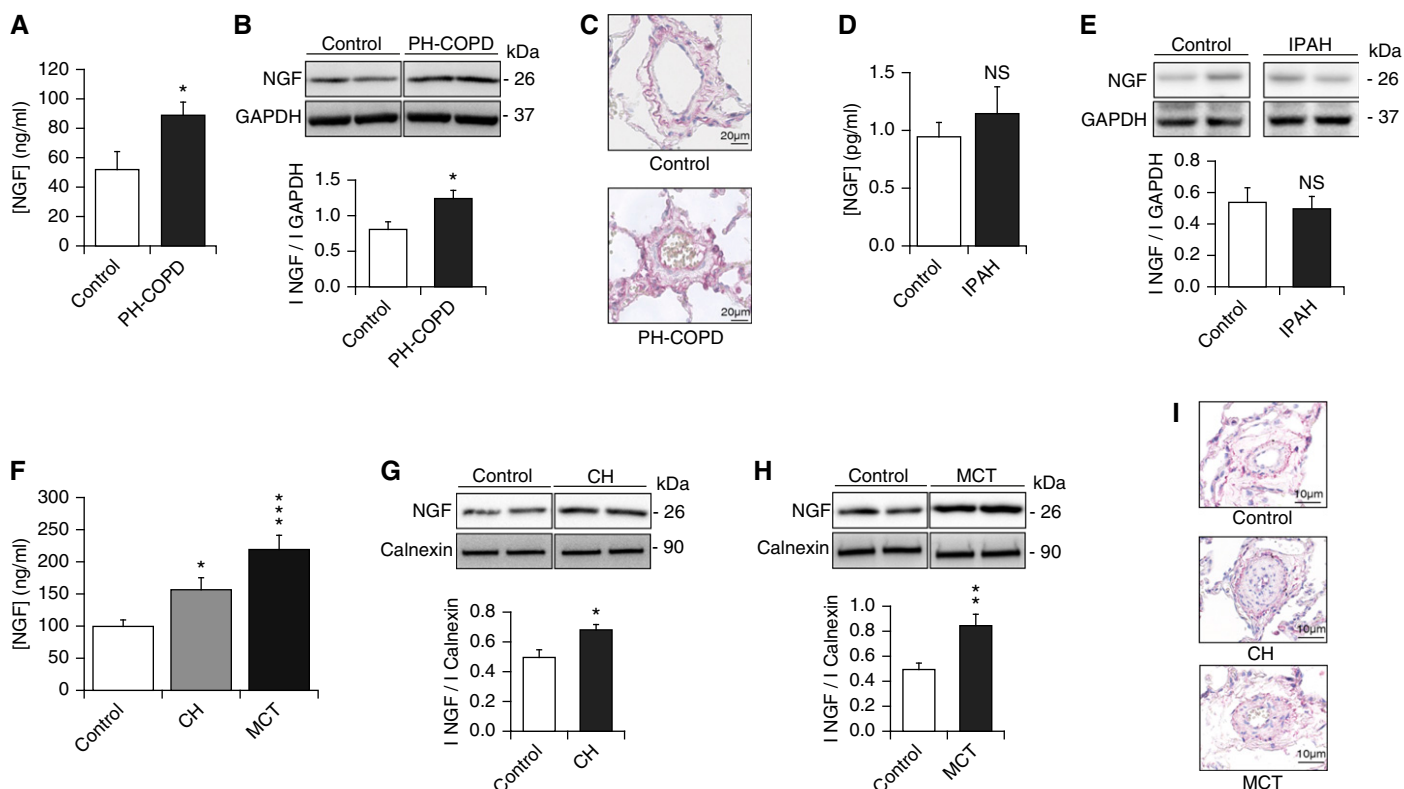


Figure 1. Nerve growth factor (NGF) secretion and expression in experimental and human pulmonary hypertension (PH). (A) NGF protein levels secreted by whole pulmonary arteries from patients with PH associated with chronic obstructive pulmonary disease (PH-COPD) compared with control patients. NGF was determined by ELISA (results expressed as ng NGF/ml supernatant after 24 h of incubation in the culture medium). Data represent means \pm SEM. $n = 5$ patients per group. * $P < 0.05$ versus control subjects. (B) Expression of NGF assessed by Western blot analysis in pulmonary arteries from patients with PH-COPD compared with control patients. Immunoblots are representative of $n = 5$ patients per group, showing identical results. Results are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and quantification of NGF expression is shown in the *bar graphs*. Data represent means \pm SEM. * $P < 0.05$ versus control subjects. (C) NGF localization assessed by immunohistochemistry in human pulmonary arteries from patients with PH-COPD compared with control patients. Stainings are representative of $n = 5$ patients in each group. (D) Circulating NGF protein levels in serum from patients with idiopathic pulmonary arterial hypertension (IPAH) compared with control patients. NGF was determined by ELISA (results expressed as pg NGF/ml serum). Data represent means \pm SEM. $n = 10$ –12 patients per group. (E) Expression of NGF assessed by Western blot analysis in whole-lung homogenates from patients with IPAH compared with control patients. Immunoblots are representative of $n = 10$ –12 patients per group, showing identical results. Results are normalized to GAPDH and quantification of NGF expression is shown in the *bar graphs*. Data represent means \pm SEM. (F) NGF protein levels secreted by whole rat pulmonary arteries in experimental PH induced either by chronic hypoxia (CH) or monocrotaline (MCT) compared with control rats. NGF was determined by ELISA (results expressed as ng NGF/ml supernatant after 24 h of incubation in the culture medium). Data represent means \pm SEM. $n = 12$ rats per group. * $P < 0.05$ and *** $P < 0.001$ versus control rats. (G and H) Expression of NGF assessed by Western blot analysis in pulmonary arteries from rats with PH induced by CH (G) and MCT (H), compared with control rats. Immunoblots are representative of $n = 6$ rats per group, showing identical results. Results are normalized to calnexin, and quantification of NGF expression is shown in the *bar graphs*. Data represent means \pm SEM. * $P < 0.05$ and ** $P < 0.01$ versus control rats. (I) NGF localization assessed by immunohistochemistry in pulmonary arteries from rats with PH induced either by CH or by MCT, compared with control animals. Stainings are representative of $n = 6$ rats in each group. NS = not statistically significant versus control subjects.

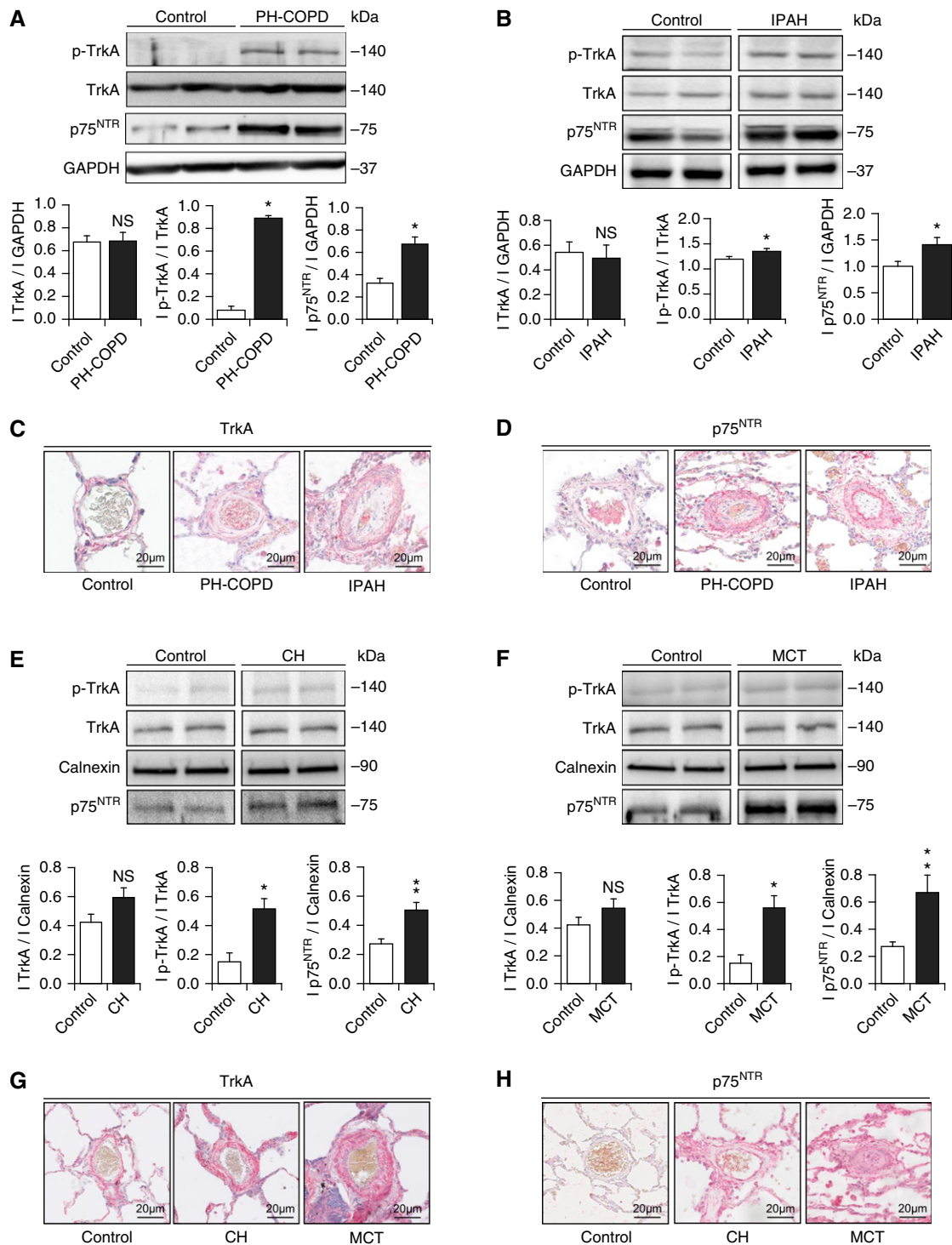


Figure 2. Expression and/or activity of nerve growth factor (NGF) receptors in experimental and human pulmonary hypertension (PH). (A and B) Expression of tropomyosin-related kinase A (TrkA) and p75^{NTR} receptors assessed by Western blot analysis in pulmonary arteries from patients with PH associated with chronic obstructive pulmonary disease (PH-COPD) (A) and in whole-lung homogenates from patients with idiopathic pulmonary hypertension (IPAH) (B), compared with control patients. TrkA phosphorylation (p-TrkA) was also assessed. Immunoblots are representative of n = 5–12 patients per group, showing identical results. Results are normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and quantification of NGF receptor expression or phosphorylation is shown in the *bar graphs*. Data represent means ± SEM. *P < 0.05 versus control patients. (C and D) Localization of TrkA (C) and p75^{NTR} (D) receptors assessed by immunohistochemistry in human pulmonary arteries from PH-COPD or patients with IPAH, compared with control patients. Stainings are representative of n = 5–6 patients in each group. (E and F) Expression of TrkA and p75^{NTR} receptors assessed by Western blot analysis in pulmonary arteries from rats with PH induced by chronic hypoxia (CH) (E) and monocrotaline (MCT) (F), compared

compared with control patients (Figures 2A–2C). However, expression of the phosphorylated TrkA receptor (on tyrosine residues) and of the p75^{NTR} receptor was significantly increased in both patients with PH-COPD and patients with IPAH compared with control subjects (Figures 2A, 2B, and 2D). Consistently, in rat experimental PH, a similar pattern of TrkA and p75^{NTR} protein expression was observed: no changes in TrkA expression but increased expressions of the TrkA phosphorylated form and of the p75^{NTR} receptor were observed in pulmonary arteries from CH-exposed and MCT-challenged rats compared with control animals (Figures 2E–2H).

Exogenous NGF Induces Pulmonary Arterial Hyperreactivity and Stimulates Pulmonary Vascular Cell Proliferation, Migration, and Secretion of Proinflammatory Cytokines

NGF by itself (up to 1 $\mu\text{g}/\text{ml}$) did not modify the basal tone of pulmonary arteries *ex vivo*. Pulmonary arterial relaxation to nitric oxide-dependent or -independent agents was not significantly modified in the presence of NGF (0–100 ng/ml, data not shown). By contrast, in the presence of NGF (100 ng/ml, 1 h of pretreatment), pulmonary arterial contraction to phenylephrine (PHE) (Figure 3A) or to prostaglandin F_{2 α} (PGF_{2 α}) (Figure 3B) was increased. Concentration–response curves were significantly shifted toward the left compared with control animals ($P < 0.01$ and $P < 0.05$ for PHE and PGF_{2 α} , respectively). Similar results were observed in pulmonary arteries from control patients with NGF pretreatment significantly increasing contractions to PHE ($P < 0.01$) or to PGF_{2 α} ($P < 0.05$) (Figures 3C and 3D).

In addition, exogenous NGF induced proliferation of hPASMCs and hPAECs *in vitro* (Figure 4). NGF proliferative effect was maximal in hPASMCs at 0.1 ng/ml ($P < 0.01$), and decreased at higher concentrations (Figure 4A). This effect was abolished when inhibiting the TrkA

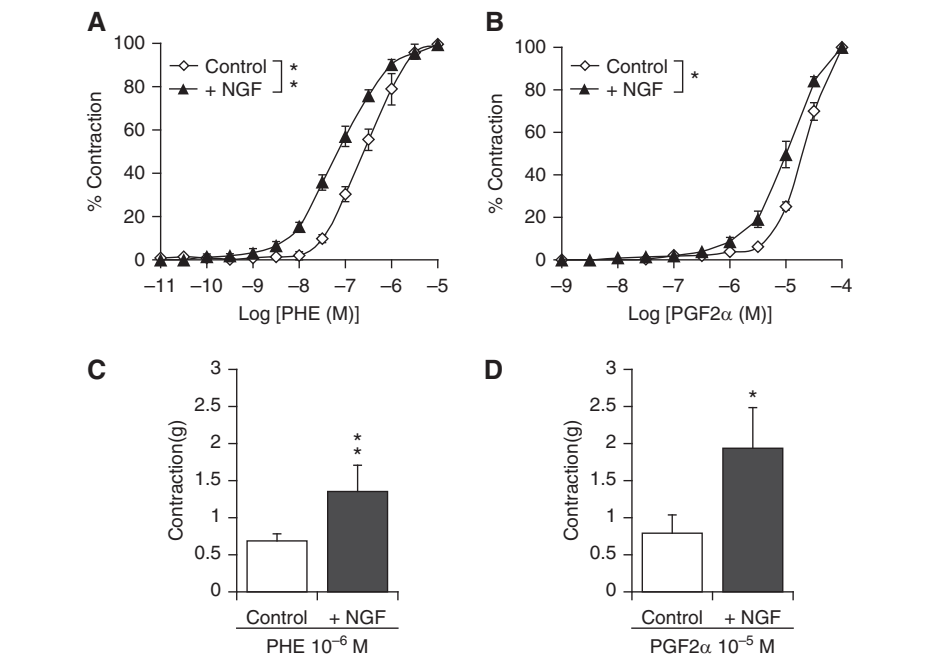


Figure 3. Effect of exogenous nerve growth factor (NGF) on rat and human pulmonary arterial reactivity. (A and B) Contractions of rat intrapulmonary arterial rings induced by cumulative concentrations of either phenylephrine (PHE; 10^{-11} to 10^{-5} mol/L) (A) or prostaglandin F_{2 α} (PGF_{2 α} ; 10^{-9} to 10^{-4} mol/L) (B). Experiments were conducted in the absence (open diamonds) or presence (solid triangles) of NGF (100 ng/ml, 1 h of pretreatment). Results are expressed as the percentage of maximal contraction and are presented as means \pm SEM. $n = 12$ rats per group. * $P < 0.05$ and ** $P < 0.01$ versus control concentration–response curves. (C and D) Contractions of human intrapulmonary arterial rings induced by a single concentration of either PHE (10^{-6} mol/L) (C) or PGF_{2 α} (10^{-5} mol/L) (D). Experiments were conducted in the absence (open bars) or presence (solid bars) of NGF (100 ng/ml, 1 h of pretreatment). Results are expressed as contractions in grams. Data represent means \pm SEM. $n = 4$ patients per group. * $P < 0.05$ and ** $P < 0.01$ versus control subjects.

receptor ($P < 0.001$), the phosphoinositide 3-kinase (PI3K) ($P < 0.001$), or the extracellular regulated-kinase (ERK) pathway ($P < 0.001$) (Figure 4A). In hPAECs, NGF proliferative effect was concentration-dependent ($P < 0.001$) and was partially blocked when inhibiting TrkA ($P < 0.05$) or p75^{NTR} receptors ($P < 0.05$). Concomitant inhibition of TrkA and p75^{NTR} receptors totally abolished NGF proliferative effect in this cell type ($P < 0.001$) (Figure 4B). In addition, NGF-induced proliferation of hPAECs was also abolished by inhibitors of atypical protein kinase Cs (PKCs) ($P < 0.001$) or of the transcription factor nuclear factor (NF)- κ B ($P < 0.001$) (Figure 4B). These results therefore show that NGF induces

proliferation of hPASMCs through TrkA-mediated activation of PI3K and ERK pathways, whereas NGF-induced proliferation of hPAECs requires both TrkA and p75^{NTR} receptors as well as the activation of distinct intracellular signaling pathways (i.e., atypical PKCs and the transcription factor NF- κ B). NGF also stimulated migration of both hPASMCs and hPAECs in a concentration-dependent manner ($P < 0.001$ for both) (see Figure E1 in the online supplement).

Finally, NGF (100 ng/ml, 24 h) increased the secretion of the proinflammatory cytokines IL-1 β ($P < 0.01$) and tumor necrosis factor- α (TNF- α) ($P < 0.001$) by control rat pulmonary arteries. Similar results were obtained in

Figure 2. (Continued). with control rats. p-TrkA was also assessed. Immunoblots are representative of $n = 6$ rats per group, showing identical results. Results are normalized to calnexin, and quantification of NGF receptor expression or phosphorylation is shown in the bar graphs. Data represent means \pm SEM. * $P < 0.05$ and ** $P < 0.01$ versus control rats. (G and H) Localization of TrkA (G) and p75^{NTR} (H) receptors assessed by immunohistochemistry in pulmonary arteries from rats with PH induced either by CH or by MCT, compared with control rats. Stainings are representative of $n = 6$ rats in each group. NS = not statistically significant versus control subjects.

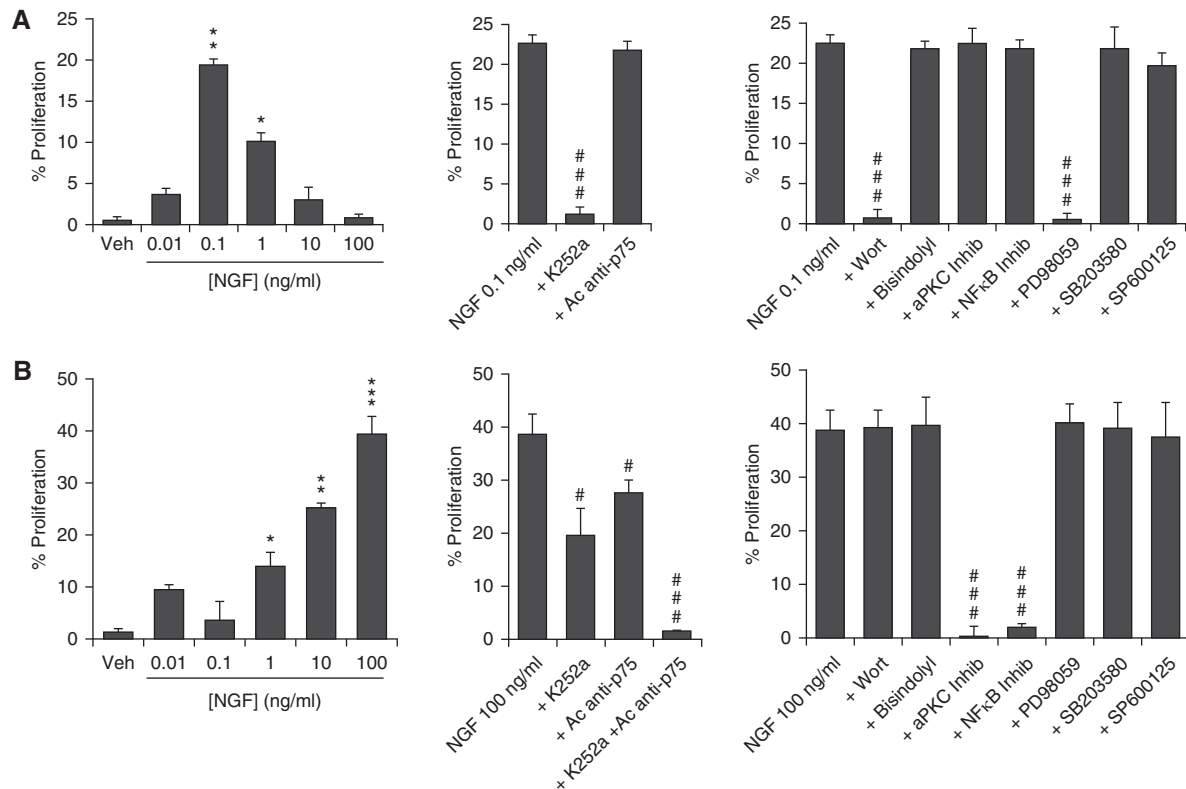


Figure 4. Proliferative effect of exogenous nerve growth factor (NGF) *in vitro* (0.01–100 ng/ml, 24 h) on human pulmonary arterial smooth muscle cells (A) or human pulmonary arterial endothelial cells (B). NGF receptors involved were identified through pretreatment (1 h) with the tropomyosin-related kinase A tyrosine-kinase inhibitor K252a (300 nM) or with an anti-p75^{NTR} blocking antibody (10 μg/ml). NGF signaling pathways involved were identified through pretreatment (1 h) with the following inhibitors: wortmannin (Wort; 100 nM, inhibiting the phosphoinositide 3-kinase), bisindolylmaleimide I (Bisindolyl; 1 μM, inhibiting conventional protein kinase Cs), PKCζ/ι pseudosubstrate inhibitor (aPKC Inhib; 10 μM, inhibiting PKCζ and PKCι atypical protein kinase Cs), nuclear factor (NF)-κB activation inhibitor (NF-κB Inhib; 100 nM, inhibiting the NF-κB), PD98059 (10 μM, inhibiting the ERK 1/2 pathway), SB203580 (2 μM, inhibiting the p38 MAP-kinase), or SP600125 (1 μM, inhibiting the JNK MAP-kinase). Data are expressed as a percentage of proliferation compared with untreated control cells and are presented as means ± SEM. n = 5 independent experiments performed in triplicate in each group. Veh = NGF vehicle. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus untreated control cells; #*P* < 0.05 and ###*P* < 0.001 versus cells treated with NGF (0.1 or 100 ng/ml).

pulmonary arteries from control patients with NGF inducing increased secretion of IL-1β (*P* < 0.01) and of TNF-α (*P* < 0.05) (Figure 5A). NGF also stimulated IL-1β and TNF-α secretion from both hPASCs and hPAECs, with a maximal effect obtained at 0.1 ng/ml of NGF in hPASCs (see Figure E2A), and at 100 ng/ml in hPAECs (see Figure E2B). In hPASCs, NGF-induced IL-1β and TNF-α secretions were both abolished by inhibition of the TrkA receptor (*P* < 0.05) (Figure 5B). NGF-induced IL-1β secretion was totally blocked by inhibitors of PI3K (*P* < 0.05) or of the ERK MAP-kinase pathway (*P* < 0.05). NGF-induced TNF-α secretion was abolished by inhibitors of conventional PKCs (*P* < 0.05), of the transcription factor NF-κB (*P* < 0.05), or of the p38 MAP-kinase (*P* < 0.05) (Figure 5A). In hPAECs, NGF-induced IL-1β and TNF-α secretions were both abolished by inhibition of the

p75^{NTR} receptor (*P* < 0.05), of conventional PKCs (*P* < 0.05), of the transcription factor NF-κB (*P* < 0.05), and of the p38 MAP-kinase (*P* < 0.05) (Figure 5C). These results therefore indicate that in hPASCs, NGF-induced secretions of both cytokines is mediated by the TrkA receptor, with distinct intracellular signaling pathways for IL-1β and TNF-α (for IL-1β, TrkA activates PI3K and ERK pathways; for TNF-α, TrkA activates conventional PKCs, p38, and NF-κB pathways). In hPAECs, both IL-1β and TNF-α secretions are mediated by the p75^{NTR} receptor, which activates conventional PKCs, p38, and NF-κB pathways.

Altogether, these *ex vivo* and *in vitro* results indicate that NGF may contribute to pulmonary arterial altered reactivity and remodeling occurring in PH, and that NGF may also play a role in inflammation

associated with PH. To validate these findings, we subsequently studied whether anti-NGF blocking antibodies administered *in vivo* would prevent and/or reverse experimental PH.

Anti-NGF Blocking Antibodies Decrease mPAP and Right Ventricular Hypertrophy in Experimental PH

Preventive protocols were conducted during 28 days, with antibodies administered from the beginning of the protocols (see Figures E3A and E3B). Curative protocols were also conducted during 28 days, but with antibodies administered only during the last 2 weeks (see Figures E3C and E3D).

PH development was evidenced by a significant increase in mPAP (*P* < 0.001) (Figures 6A and 6B) and by a right ventricular hypertrophy (Fulton index, *P* < 0.001) (Figures 6C and 6D) compared with control subjects. Preventive or curative

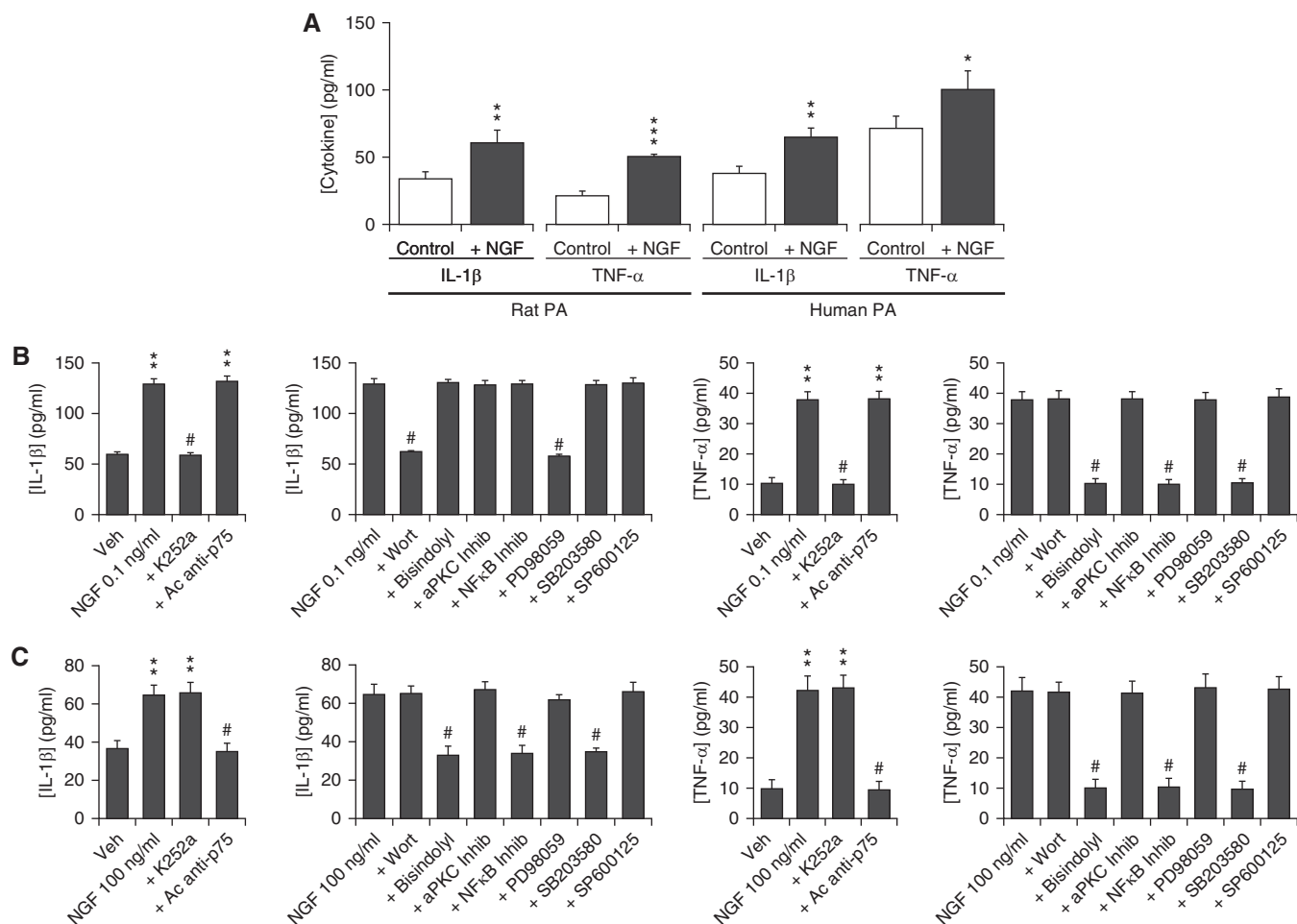


Figure 5. Effect of exogenous nerve growth factor (NGF) *ex vivo* and *in vitro* on secretion of inflammatory cytokines. (A) Secretions of the proinflammatory cytokines IL-1 β and tumor necrosis factor- α (TNF- α) by rat or human pulmonary arteries (PA), in the absence (*open bars*) or presence (*solid bars*) of NGF (100 ng/ml, 24 h). Cytokines were determined by ELISA (results expressed as pg cytokine/ml supernatant after 24 h of incubation in the culture medium). Data represent means \pm SEM. $n = 12$ rats or $n = 4$ patients per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control subjects. (B and C) Secretions of IL-1 β and TNF- α induced by NGF (0.1 or 100 ng/ml, 24 h) in human pulmonary arterial smooth muscle cells (B) or human pulmonary arterial endothelial cells (C). NGF receptors involved were identified through pretreatment (1 h) with the tropomyosin-related kinase A tyrosine-kinase inhibitor K252a (300 nM) or with an anti-p75^{NTR} blocking antibody (10 μ g/ml). NGF signaling pathways involved were identified through pretreatment (1 h) with the following inhibitors: wortmannin (Wort; 100 nM, inhibiting the phosphoinositide 3-kinase), bisindolylmaleimide I (Bisindolyl; 1 μ M, inhibiting conventional protein kinase Cs), PKC ζ / ι pseudosubstrate inhibitor (aPKC Inhib; 10 μ M, inhibiting the atypical PKC ζ and PKC ι protein kinase Cs), nuclear factor (NF)- κ B activation inhibitor (NF- κ B Inhib; 100 nM, inhibiting the NF- κ B), PD98059 (10 μ M, inhibiting the ERK 1/2 pathway), SB203580 (2 μ M, inhibiting the p38 MAP-kinase), or SP600125 (1 μ M, inhibiting the JNK MAP-kinase). Cytokines were determined by ELISA (results expressed as pg cytokine/ml supernatant after 24 h of incubation). Data represent means \pm SEM. $n = 5$ independent experiments performed in triplicate in each group. Veh = NGF vehicle. ** $P < 0.01$ versus untreated control cells; # $P < 0.05$ versus cells treated with NGF (0.1 or 100 ng/ml).

treatment with nonrelevant antibodies did not affect values of mPAP and of Fulton index. However, in both CH- and MCT-induced PH, animals preventively or curatively treated with anti-NGF blocking antibodies displayed lower mPAP (Figures 6A and 6B) and Fulton index values (Figures 6C and 6D). Our results therefore suggest that NGF is involved in both PH development and persistence. To better understand the beneficial effects of NGF inhibition in PH, we further investigated

the pathophysiologic mechanisms involved.

Anti-NGF Blocking Antibodies Decrease Pulmonary Arterial Hyperreactivity, Remodeling, and Inflammation in Experimental PH

Intrapulmonary arteries from CH-exposed (Figure 7A) or MCT-treated rats (Figure 7B) displayed hyperreactivity to PHE, with concentration-response curves significantly shifted toward the left

compared with control animals ($P < 0.01$ for both). Hyperreactivity to PGF_{2 α} was also observed in both PH rat models (see Figures E4A and E4B). Administration of nonrelevant antibodies had no significant effect on these shifts. By contrast, treatment with anti-NGF blocking antibodies partially reversed (curative protocol, $P < 0.05$) (Figures 7A and 7B) or totally prevented hyperreactivity to PHE (preventive protocol, $P < 0.01$) (see Figures E5A and E5B). Similar results were observed for

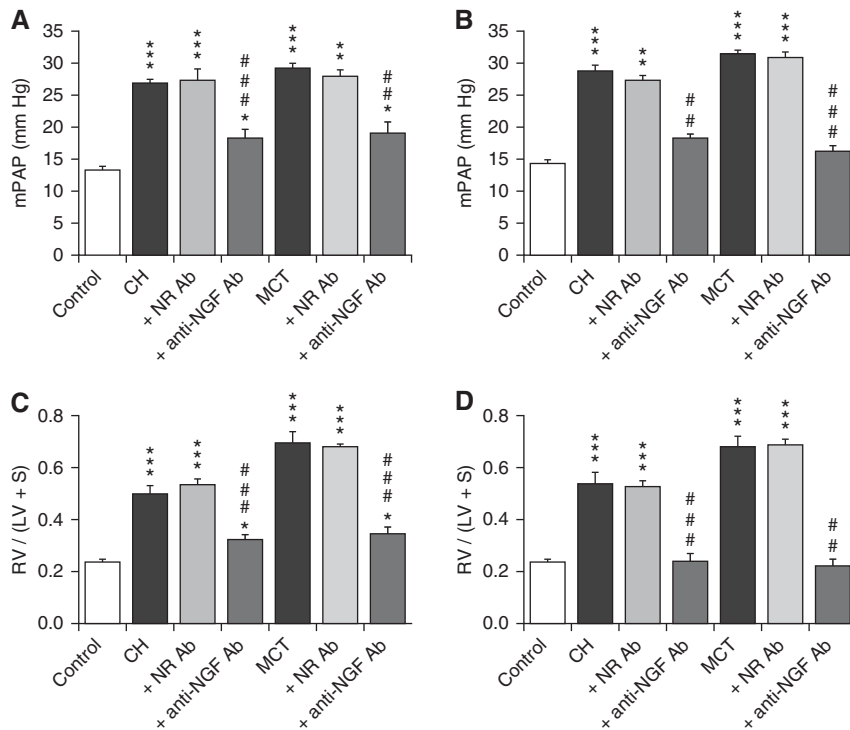


Figure 6. Prevention and reversal of increased mean pulmonary arterial pressure (mPAP) and right ventricular hypertrophy by anti-nerve growth factor (NGF) blocking antibodies in hypoxia- and monocrotaline-induced pulmonary hypertension. (A and B) mPAP (in mm Hg) measured on Day 28 in control rats, after chronic hypoxia (CH) or after monocrotaline treatment (MCT), and in rats exposed to CH or to MCT and treated either preventively (A) or curatively (B) with nonrelevant (+ NR Ab) or with anti-NGF blocking (+ anti-NGF Ab) antibodies. Data represent means \pm SEM. $n = 8-12$ rats per group. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus control animals; $##P < 0.01$ and $###P < 0.001$ versus CH or MCT. (C and D) Right ventricular hypertrophy expressed as the Fulton index (= ratio of right ventricle weight [RV] to left ventricle plus septum weight [LV + S]) in the same experimental groups in preventive (C) or curative (D) protocols. Data represent means \pm SEM. $n = 8-12$ rats per group. $*P < 0.05$ and $***P < 0.001$ versus control animals; $##P < 0.01$ and $###P < 0.001$ versus CH or MCT.

PGF_{2 α} , with hyperreactivity to PGF_{2 α} being partially reversed in the curative protocol (see Figures E4A and E4B), and totally prevented in the preventive protocol (see Figures E5C and E5D).

Hyperreactivity to PHE was confirmed *in vivo* in both PH rat models by direct injection of PHE into the main pulmonary artery. In control rats, PHE induced a transient increase in mPAP (Figure 7C; see Figure E4C). No differences were observed in rats treated with anti-NGF blocking antibodies compared with control rats. PHE-induced increase in mPAP was significantly greater in CH-exposed ($P < 0.01$) or MCT-challenged rats ($P < 0.05$) than in control rats. This increase was significantly reduced in rats exposed to CH or to MCT and curatively treated with anti-NGF blocking antibodies ($P < 0.05$ for both) (Figure 7C; see Figure E4C). Therefore, both our *ex vivo* and *in vivo* data

strongly suggest a role for NGF in pulmonary arterial hyperreactivity during PH.

A role of NGF inhibition in pulmonary arterial remodeling *in vivo* was studied by quantitative analysis of medial wall thickness in intrapulmonary arterioles (external diameter $< 50 \mu\text{m}$). Compared with control subjects, this parameter was significantly increased in both CH-exposed and MCT-treated rats ($P < 0.01$ for both) (Figure 8). Administration of nonrelevant antibodies had no significant effect on this medial thickening. By contrast, administration of anti-NGF blocking antibodies partially reduced values of medial wall thickness in lungs of CH-exposed ($P < 0.05$) or of MCT-treated rats ($P < 0.01$). These results were confirmed by α -smooth muscle actin immunostaining, showing that anti-NGF blocking antibodies reduced accumulation

of pulmonary smooth muscle cells (Figure 8B). In the preventive protocol, medial thickening was totally prevented by administration of anti-NGF blocking antibodies in both CH-exposed and MCT-treated rats (see Figure E6A).

Finally, we studied the effects of NGF blockade on inflammation associated with PH. We showed that the secretion of proinflammatory cytokines by pulmonary arteries was significantly increased in CH-exposed or MCT-treated rats ($P < 0.001$) (Figure 9A). This increase was not significantly modified when rats were treated with nonrelevant antibodies, but was partially or totally reversed when rats were treated with anti-NGF blocking antibodies in the curative protocol ($P < 0.01$ or 0.001 vs. CH or MCT) (Figure 9A). In the preventive protocol, IL-1 β and TNF- α increased secretions were also partially or totally prevented in both CH-exposed and MCT-treated rats (see Figure E6C). Finally, the effects of NGF inhibition on the recruitment of inflammatory cells in lung tissues were also evaluated. After CH exposure or MCT challenge, Western blot analysis of lung homogenates showed increased protein expression of CD45, a marker of inflammatory cell infiltration in the lung parenchyma ($P < 0.01$) (Figure 9B). Treatment with nonrelevant antibodies had no significant effect on this parameter. By contrast, CD45-increased expression was totally reversed in CH-exposed rats and partially reversed in MCT-treated rats after administration of anti-NGF blocking antibodies (Figure 9B). Similar effects were observed in the preventive protocol (see Figure E6B).

Discussion

In this study, we describe for the first time an increase in expression of NGF and/or in expression/activation of its receptors in experimental and human PH, supporting a critical role for NGF and its signaling pathways in this disease. Experiments conducted *in vitro* and *ex vivo* demonstrated that NGF induces (1) hyperreactivity to vasoconstrictors in rat and human isolated pulmonary arteries, (2) proliferation and migration of hPASMCs and hPAECs, and (3) secretion of the proinflammatory cytokines IL-1 β and TNF- α from rat and human isolated pulmonary arteries as well as from

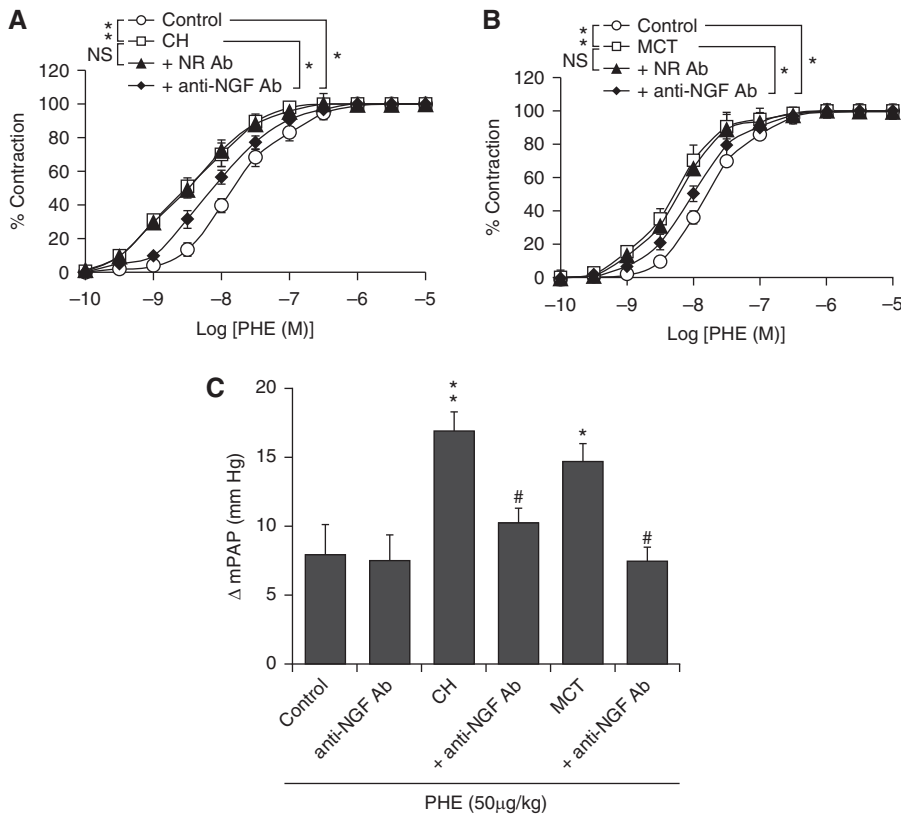


Figure 7. Reversal of pulmonary arterial hyperreactivity by anti-nerve growth factor (NGF) blocking antibodies in hypoxia- and monocrotaline-induced pulmonary hypertension. (A and B) *Ex vivo* contractions of rat intrapulmonary arterial rings induced by cumulative concentrations of phenylephrine (PHE; 10^{-10} to 10^{-5} mol/L). Contractions were studied after killing on Day 28 in control rats, after chronic hypoxia (CH), or after monocrotaline treatment (MCT), and in rats exposed to CH or to MCT and curatively treated with nonrelevant (+ NR Ab) or with anti-NGF blocking (+ anti-NGF Ab) antibodies. Data represent means \pm SEM. $n = 8-12$ rats per group. NS = not statistically significant; $*P < 0.05$ and $**P < 0.01$ versus control animals, CH or MCT concentration-response curves. (C) *In vivo* measurements of mean pulmonary arterial pressure variations (Δ mPAP in mm Hg) after direct injection of PHE (50 μ g/kg) in the main pulmonary artery. mPAP were measured on Day 28 in control rats, in rats treated with anti-NGF blocking antibodies (anti-NGF Ab), after CH or MCT treatment, and in rats exposed to CH or to MCT and curatively treated with anti-NGF blocking antibodies (+ anti-NGF Ab). Data represent means \pm SEM. $n = 4-5$ rats per group. $*P < 0.05$ and $**P < 0.01$ versus control animals; $\#P < 0.05$ versus CH or MCT.

hPASMCs and hPAECs. In addition, *in vivo* experiments showed that NGF inhibition by anti-NGF blocking antibodies administered to CH-exposed or MCT-challenged rats not only prevents but also reverses PH. In these *in vivo* experiments, NGF inhibition decreased pulmonary vascular remodeling and limited pulmonary arterial hyperreactivity and inflammation. Therefore, our findings suggest that NGF may represent a target of particular interest in future PH therapeutic strategy.

In this study, NGF and its receptors were expressed in pulmonary arteries from control rats or from control patients, in accordance with the literature (14, 25) and with previous results from our group (26).

We show here that NGF expression was increased in pulmonary arteries in experimental PH induced by either CH exposure or MCT challenge. Such overexpression was confirmed in patients with PH-COPD, but not in patients with IPAH. Although an increase in NGF expression has been previously identified in spontaneously hypertensive rat lungs (27), this is the first report of NGF overexpression in PH. Differences in NGF expression observed in patients with PH-COPD versus with IPAH may be explained by the different pathophysiologic mechanisms involved in these two forms of PH. In particular, CH induces specific cellular and molecular mechanisms, which

trigger inflammation and pulmonary vascular remodeling (28). In addition, there is increasing evidence to support a role of reactive oxygen species in CH PH (28-30).

Hypoxia and oxidative stress have been shown to increase NGF expression in various tissues (31, 32) so they may therefore contribute to NGF overexpression observed in PH-COPD. Inflammation may also play an important role in this process. Inflammatory cells, such as lymphocytes, macrophages, or mast cells, have been shown to produce NGF (18). These inflammatory cells infiltrated in and around pulmonary arteries could therefore be a source of NGF in PH. Moreover, NGF expression is up-regulated under inflammatory conditions in airway structural cells (18, 33, 34). Pulmonary vascular cells can secrete NGF (26), and may also contribute to NGF increased expression in inflammatory conditions (35-38). However, pulmonary inflammation differs according to the form of PH, and different inflammatory mechanisms may therefore also contribute to the differences in NGF expression observed in patients with PH-COPD versus patients with IPAH (28, 37). Finally, because the different IPAH samples used in this study were obtained from patients with end-stage IPAH receiving drug therapy, we cannot exclude that NGF overexpression may vary between stages of the disease and may also be influenced by treatments.

Our results also show increased expression and/or activity of both NGF receptors (TrkA and p75^{NTR}) in human and experimental PH. The TrkA receptor belongs to the family of receptor tyrosine-kinases (RTKs), sharing with them a similar mode of activation (39). Involvement of other RTKs, including platelet-derived growth factor receptor, fibroblast growth factor receptor, or c-kit, has already been described in pulmonary vascular remodeling (8, 10, 11, 40-42). In our study, TrkA protein expression was not different in patients with PH compared with control patients. However, TrkA phosphorylation on tyrosine residues was significantly increased in patients with PH-COPD and patients with IPAH, suggesting increased TrkA activity. Such increase was also observed in the two PH rat models used in this study. NGF expression, when elevated in PH-COPD pulmonary arteries, may contribute to TrkA increased activation. In addition, hypoxia can down-regulate

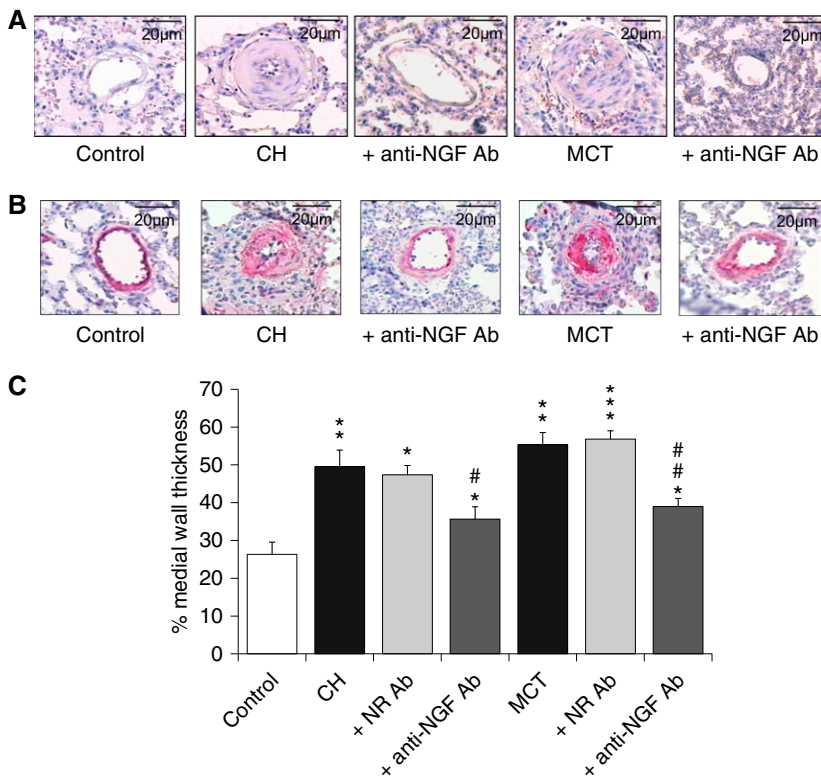


Figure 8. Reversal of increased medial wall thickness by anti-nerve growth factor (NGF) blocking antibodies in hypoxia- and monocrotaline-induced pulmonary hypertension. (A and B) Representative sections of small pulmonary arteries from the different experimental groups stained with hematoxylin and eosin (A) or immunostained for α -smooth muscle actin (B). (C) Percentage of medial wall thickness of small pulmonary arteries (external diameter <math>< 50 \mu\text{m}</math>) in control rats, after chronic hypoxia (CH) or monocrotaline treatment (MCT), and in rats exposed to CH or MCT and curatively treated with nonrelevant (+ NR Ab) or anti-NGF blocking (+ anti-NGF Ab) antibodies. Data represent means \pm SEM. $n = 8\text{--}12$ rats per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus control animals; # $P < 0.05$ and ## $P < 0.01$ versus CH or MCT.

expression of numerous protein tyrosine phosphatases, therefore leading to RTKs enhanced activity (43). Because PH-COPD is usually associated with hypoxemia, such mechanism may also occur for the TrkA receptor and needs to be further investigated.

Alternative mechanisms increasing TrkA phosphorylation have been described even in the absence of increased NGF expression. In particular, changes in the composition of plasma membrane lipids can potentiate NGF-mediated TrkA activation, or even activate the TrkA receptor in the absence of NGF (44). Altered membrane lipid domains have been described in PH (45) and may thus contribute to TrkA-enhanced activity in IPAH lungs. Our results also show increased expression of the $p75^{\text{NTR}}$ receptor in human and experimental PH. This receptor belongs to the death receptors family (39), but may

paradoxically contribute to pulmonary vascular remodeling, as recently shown for the TNF-related apoptosis-inducing ligand (46). Taken together, our findings highlight the fact that NGF and/or its signaling pathways are deregulated in experimental and human PH. This may therefore contribute to the progression of the disease in patients with PH-COPD and patients with IPAH, but probably through different mechanisms that remain to be determined. These results also suggest that targeting either NGF or its receptors may offer different therapeutic benefits in patients with PH-COPD and patients with IPAH.

Because expression of NGF and/or expression/activation of its receptors are increased in PH, we further investigated the potential role of NGF in this disease using two animal models of PH. Our results show that anti-NGF blocking antibodies prevent and reverse PH, as reflected by decreased

values of mPAP and right ventricular hypertrophy. These results suggest that NGF plays critical roles in PH development and persistence. Thus, we further investigated the role of NGF in three key pathophysiologic features of PH: (1) pulmonary arterial altered reactivity, (2) remodeling, and (3) inflammation.

First, we observed that NGF induced hyperreactivity of rat and human pulmonary arteries to contractile agents *ex vivo*. Consistently, we demonstrated in both *ex vivo* and *in vivo* studies that anti-NGF blocking antibodies totally prevented (preventive protocol) and partially reversed (curative protocol) pulmonary arterial hyperreactivity in CH-exposed or MCT-challenged rats. Other studies have already shown that NGF induces airway hyperreactivity in both animal and human tissues (18, 47), but this is the first report of NGF-induced vascular hyperreactivity, whatever the tissue considered. Such modulation of pulmonary reactivity by NGF is in accordance with a previous study showing that the $p75^{\text{NTR}}$ receptor may be an important regulator of pulmonary vascular tone (48). In addition, other neurotrophins, such as brain-derived neurotrophic factor or neurotrophin-3, have also been shown to regulate pulmonary vascular reactivity, by inducing pulmonary endothelial nitric oxide production and relaxation (49). By contrast, in our experiments, NGF by itself did not produce any effect on pulmonary arteries. In addition, the relaxation of rat pulmonary arteries induced by nitric oxide-dependent or -independent agents was not modified in the presence of NGF. These results therefore suggest that in pulmonary arteries, effects and underlying signaling pathways differ depending on the neurotrophin considered.

In other tissues, NGF has been found to induce a rapid relocalization of calcium channels at the cell membrane (50) and can inhibit potassium channels (51). NGF is also known to augment neuronal responsiveness to noradrenaline by increasing expression of the α -adrenergic receptor (52), and can activate the RhoA/ROCK pathway through activation of the $p75^{\text{NTR}}$ receptor (53). Such NGF-dependent mechanisms, if occurring in pulmonary arteries, may contribute to the altered pulmonary arterial reactivity in PH.

We previously demonstrated that NGF induced proliferation and migration of

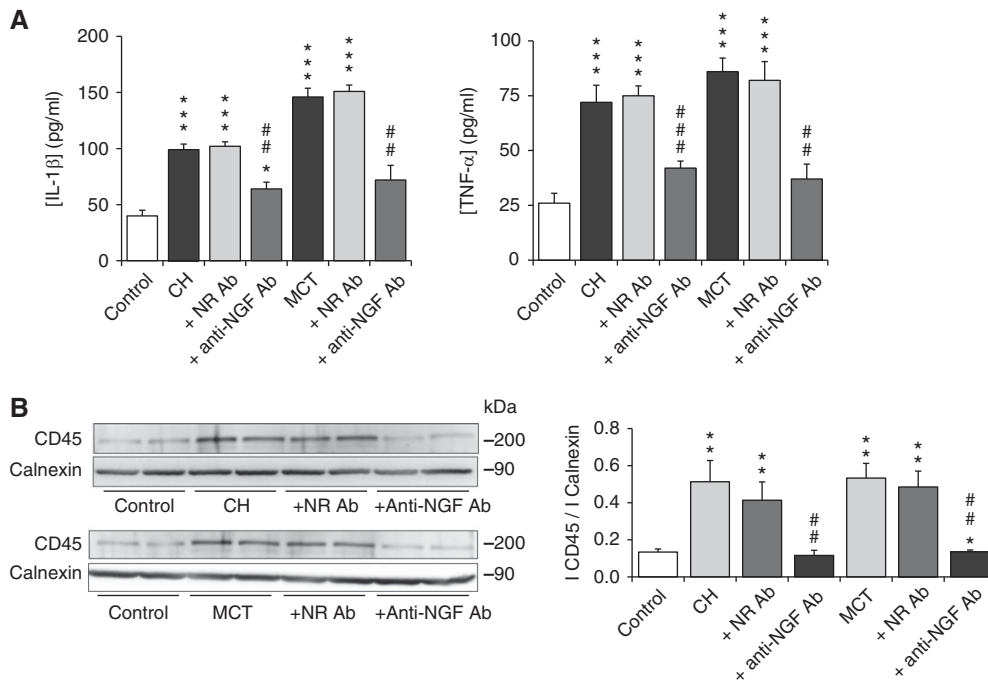


Figure 9. Reversal of inflammation by anti-nerve growth factor (NGF) blocking antibodies in hypoxia- and monocrotaline-induced pulmonary hypertension. (A) Secretions of the proinflammatory cytokines IL-1 β and tumor necrosis factor- α (TNF- α) by pulmonary arteries in control rats, after chronic hypoxia (CH) or monocrotaline treatment (MCT), and in rats exposed to CH or MCT and curatively treated with nonrelevant (+ NR Ab) or anti-NGF blocking (+ anti-NGF Ab) antibodies. Cytokines were determined by ELISA (results expressed as pg cytokine/ml supernatant after 24 h of incubation in the culture medium). Data represent means \pm SEM. $n = 8$ –12 rats per group. * $P < 0.05$ and *** $P < 0.001$ versus control animals; ## $P < 0.01$ and ### $P < 0.001$ versus CH or MCT. (B) CD45 quantification assessed by Western blot analysis in whole-lung homogenates from the same experimental groups. Immunoblots are representative of $n = 6$ rats per group, showing identical results. CD45 expression is normalized to calnexin. Quantification of CD45 expression is shown in the *bar graphs*, with data representing means \pm SEM. * $P < 0.05$ and ** $P < 0.01$ versus control animals; ## $P < 0.01$ versus CH or MCT.

hPASMCs and hPAECs *in vitro* (26). These results are in accordance with the ability of NGF to induce proliferation and/or migration of endothelial or smooth muscle cells from various tissues (15, 16, 54, 55). They are also in accordance with a recent study showing that brain-derived neurotrophic factor augments smooth muscle cell proliferation in pulmonary arteries (56). Interestingly, NGF-induced maximal proliferation occurred at low NGF concentrations in hPASMCs, and our results support a role of the TrkA receptor in this effect via activation of PI3K and ERK pathways. The maximal proliferative effect observed at 0.1 ng/ml of NGF is in accordance with NGF picomolar affinity for the TrkA receptor, and ERK pathway has also been shown to be implicated in TrkA-induced proliferation of human airway smooth muscle cells (15). Surprisingly, NGF-induced hPAEC proliferation requires both TrkA and p75^{NTR} receptors, activating atypical PKCs and the transcription factor NF- κ B. The maximal proliferative effect in hPAECs

observed at 100 ng/ml of NGF is in accordance with a role of the p75^{NTR} receptor, because NGF has a lower affinity for this receptor. Altogether, these results therefore support a role of NGF in pulmonary vascular remodeling associated with PH, through activation of both TrkA and p75^{NTR} receptors that lead to distinct intracellular signaling pathways in hPASMCs and hPAECs.

Consistently, NGF inhibition *in vivo* prevented (preventive protocol) and reversed (curative protocol) increased thickness of the pulmonary arterial media, in particular smooth muscle accumulation. Because proliferation and migration of PASMCs and PAECs are major features contributing to pulmonary vascular remodeling through medial and intimal hypertrophy (7), these results strongly suggest that NGF participates in pulmonary arterial remodeling, in particular smooth muscle remodeling *in vivo*.

Finally, we also showed that exogenous NGF triggers the secretion of IL-1 β and TNF- α by rat and human pulmonary

arteries *ex vivo*, as well as by hPASMCs and hPAECs *in vitro*. Furthermore, we demonstrated that activation of NGF receptors and their intracellular signaling pathways depends on the type of cell and on the cytokine of interest. In hPASMCs, NGF-induced cytokine secretion involves the TrkA receptor, with activation of PI3K and ERK pathways for IL-1 β and with activation of conventional PKCs, p38, and NF- κ B pathways for TNF- α secretion. In hPAECs, NGF-induced cytokine secretion involves the p75^{NTR} receptor, with activation of conventional PKCs, p38, and NF- κ B pathways. As for cell proliferation, the maximal cytokine secretion in hPASMCs is observed at 0.1 ng/ml of NGF, in accordance with NGF picomolar affinity for the TrkA receptor. In contrast, the maximal cytokine secretion in hPAECs is observed at 100 ng/ml of NGF, in accordance with a role of the NGF low-affinity p75^{NTR} receptor in this effect. Altogether, these results therefore show that NGF, activating both TrkA and p75^{NTR} receptors, but through distinct

signaling pathways, may contribute to pulmonary vascular inflammation associated with PH.

Consistently, anti-NGF blocking antibodies have been found to inhibit IL-1 β and TNF- α increased secretions by pulmonary arteries and to decrease the infiltration of inflammatory cells in lung tissues from CH-exposed or MCT-treated rats, both in preventive and in curative protocols. Levels of proinflammatory cytokines, such as IL-1 β and TNF- α , are known to be increased in human and experimental PH, not only in the systemic circulation, but also locally in pulmonary arteries (38, 57, 58). A proinflammatory role of NGF has already been shown in airway inflammatory diseases, such as asthma (18). NGF can stimulate various

inflammatory cells to trigger their recruitment to inflamed tissues, activation, and survival (59–62). In accordance with these studies, our *in vivo* results argue in favor of a role of NGF in PH-associated inflammation, by stimulating the production of IL-1 β and TNF- α by pulmonary vascular cells and by recruiting inflammatory cells in lung tissues.

In conclusion, we demonstrate in this study that targeting NGF may be an interesting novel therapeutic strategy in PH treatment. Indeed, our *in vitro*, *ex vivo*, and *in vivo* findings consistently indicate that NGF inhibition not only decreases pulmonary vascular remodeling, but also decreases pulmonary arterial hyperreactivity and inflammation, which

are all important features of PH. The recent development of several humanized anti-NGF blocking antibodies (i.e., tanezumab and fasinumab) to treat pain associated with osteoarthritis (63, 64), acute sciatic (65), or chronic low back pain (66) may therefore rapidly open new perspectives in the treatment of PH. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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