Role of Src Tyrosine Kinases in Experimental Pulmonary Hypertension

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Objective—Pulmonary arterial hypertension is a progressive pulmonary vascular disorder with high morbidity and mortality. Compelling evidence suggests that receptor tyrosine kinases, such as platelet-derived growth factor (PDGF) are closely involved in the pathogenesis of pulmonary arterial hypertension. We investigated the effects of 2 novel PDGF inhibitors, nilotinib/AMN107 (Abl kinases/PDGF receptor inhibitor) and dasatinib/BMS-354825 (Abl kinases/PDGF receptor/Src inhibitor), on the proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) and on the hemodynamics and pulmonary vascular remodeling in experimental pulmonary hypertension, and determined the expression and regulation of Src family kinases.

Methods and Results—Human PASMCs were stimulated by PDGF alone or multiple growth factors to induce proliferation and migration in vitro. Dasatinib (0.03 μmol/L), nilotinib (0.3 μmol/L), and imatinib (1 μmol/L) potently inhibited PDGF-induced signal transducer and activator of transcription 3 and Akt phosphorylation. All 3 inhibitors decreased PDGF-induced proliferation, cell cycle gene regulation, and migration. In contrast, only dasatinib inhibited multiple growth factor–induced PASMC proliferation, and this was associated with the inhibition of Src phosphorylation. Combination of specific Src inhibitors (phosphoprotein phosphatase 1, phosphoprotein phosphatase 2) with either imatinib or nilotinib reduced multiple growth factor–induced proliferation to a similar extent as dasatinib. Importantly, Src phosphorylation increased in pulmonary arterial hypertension PASMCs compared with control PASMCs. Finally, in vivo dasatinib (15 mg/kg per body weight) treatment caused a complete reversal of pulmonary vascular remodeling and achieved similar effectiveness as imatinib (100 mg/kg per body weight) in both monocrotaline- and hypoxia-induced pulmonary hypertension models.

Conclusion—We suggest that dual inhibition of PDGF receptor and Src kinases potently inhibits mitogenic and motogenic responses to growth factors in PASMCs and pulmonary vascular remodeling in vivo so that dual inhibition may represent an alternative therapeutic approach for pulmonary arterial hypertension. (Arterioscler Thromb Vasc Biol. 2012;32:1354-1365.)

Key Words: pulmonary hypertension • smooth muscle • vasculature

Pulmonary arterial hypertension (PAH) is a progressive pulmonary vascular disorder with high morbidity and mortality.1 Current therapeutic approaches for the treatment of PAH mainly provide symptomatic relief, as well as some improvement in prognosis. Pathological changes observed in vascular remodeling include endothelial injury, proliferation, and migration of pulmonary arterial smooth muscle cells (PASMCs), causing medial hypertrophy of the intracinar muscular resistance vessels, muscularization of the normally nonmuscular precapillary arterioles, and an increase in connective tissue deposition in the medial layer.2 Over recent years, considerable evidence has been accumulated suggesting the involvement of receptor tyrosine kinases (RTK), such as the platelet-derived growth factor (PDGF) receptor, in the pathogenesis of pulmonary vascular remodeling. A complete analysis of the pathogenic role of PDGF in PAH demonstrated an increased expression of PDGF ligands and receptors (PDGFRβ) in pulmonary arteries of idiopathic PAH lungs.3,4 In addition, PDGF was shown to primarily contribute to the proliferative and migratory phenotype of PASMCs.5 Administration of imatinib, a small molecular inhibitor that inhibits PDGFR/Ab1 and c-Kit,6 was shown to reverse vascular remodeling in severe experimental...
pulmonary hypertension (PH). Patients with severe PAH, who failed to be stabilized even by extensive combined application of approved PAH therapies, were beneficially influenced by compassionate use of imatinib as rescue therapy.7,8

Inhibition of PDGFR by imatinib, however, requires micromolar concentrations in cell-based assays.9,10 Similarly, higher concentrations are required to achieve reverse remodeling effects in preclinical models of PH.5 This prompted us to search for other tyrosine kinase inhibitors that can potently inhibit PDGFR/Abl tyrosine kinases. In addition, activation of epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin growth factor (IGF), and insulin receptors was shown to contribute to PASMC proliferative responses.11 These findings suggest that inhibitors that can target a common downstream signaling molecule of RTKs can be of potential therapeutic value.

Nilotinib (AMN107) and dasatinib (BMS-354825) are 2 novel inhibitors of PDGFR/Abl and c-Kit (so-called second-generation RTK inhibitors), which have shown higher potency compared with imatinib in in vitro kinase assays.12,13 Importantly, dasatinib apart from PDGFR/Abl and c-Kit also inhibits the structurally related Src family kinases (SFKs).14 These 2 compounds are in clinical development for oncology indications.13

SFKs are the largest subfamily of non-RTKs consisting of 9 kinases, namely Src (c-Src), Yes, Fyn, Blk, Lyn, Hck, Yrs, Lck, Fgr, which share similar structures and function.15 SFKs play an important role in regulating signals from many RTKs and have evolved many ingenious molecular strategies to couple receptors with the cytoplasmic signaling machinery. Thus SFKs can promote mitogenic signaling from RTKs in many ways, including initiation of signaling pathways required for DNA synthesis, control of receptor turnover, differentiation, actin cytoskeleton rearrangements, motility, and survival.16,17 However, contribution of SFKs to vascular remodeling and to the pathogenesis of PAH has not been explored yet. Because dasatinib inhibits SFKs, it would be interesting to know how dasatinib regulates SFKs in PAH and in turn pulmonary vascular remodeling.

We sought to (1) determine the influence of nilotinib and dasatinib on PDGF signaling and PDGF-stimulated migration and proliferation of PASMCs compared with imatinib; (2) study whether imatinib, nilotinib, and dasatinib can inhibit multiple growth factor (MGF)-induced proliferation and migration; (3) study the expression, regulation, and contribution of SFKs in PAH; (4) analyze the influence of dasatinib, imatinib, and nilotinib on Src tyrosine kinase activity; and (5) compare the reverse remodeling potency exerted by dasatinib, nilotinib, and imatinib in experimental PH.

Materials and Methods

MCT Treatment, Inhibitor Treatment, and Hemodynamic Measurements

Local ethics committees approved all animal experiments. Rats were injected with 60 mg/kg monocrotaline (MCT) subcutaneously. Twenty-one days after a single MCT injection, rats were randomized to receive daily dasatinib (5 mg/kg per body weight [bw] or 15 mg/kg per bw), nilotinib (30 mg/kg per bw), imatinib (100 mg/kg per bw), or placebo (2% Methocell) orally by gavage for 14 days. After 5 weeks, hemodynamics, cardiac output, and right ventricular (RV) hypertrophy were assessed (detailed protocol is available in the online-only Data Supplement).

Assessment of Vascular Remodeling and Proliferation In Situ

Paraffin-embedded lung sections were double stained with α-smooth muscle actin and von Willebrand factor antibodies for assessing the degree of muscularization of peripheral pulmonary arteries. In addition, lung sections were stained for Elastin–Nuclear Fast Red to assess medial wall thickness. Vessels were detected by staining for von Willebrand factor, proliferation by staining for proliferating cell nuclear antigen (PCNA), and apoptosis by staining with an in situ cell death detection kit.

Cell Culture

Human PASMCs (hPASMCs) were obtained from Lonza. Primary rat PASMCs were isolated from control and MCT-induced PH (MCT-PH) rats by explant method.

Proliferation Assays

Influence of dasatinib, nilotinib, and imatinib on PDGF-BB-induced or MGF-induced (5% fetal calf serum, PDGF-BB 30 ng/mL, FGF 2 ng/mL, EGF 0.5 mg/mL, insulin 0.5 μg/mL) PASMC proliferation was assessed using a bromodeoxyuridine (BrdU) incorporation assay, where the rates of DNA synthesis were used as a marker of cellular proliferation. The concentrations of the inhibitors were selected on the basis of previous publications.

Chemotaxis Assays

Influence of dasatinib, nilotinib, and imatinib on PDGF-BB- or MGF-induced PASMC chemotaxis was assayed using a modified Boyden chemotaxis chamber. Chemotaxis was quantified by counting the number of cells on the lower surface of the filter in each well using a grid containing 100 nonoverlapping fields.

Immunocytochemistry

hPASMCs were grown on chamber slides and were fixed immediately in acetone and methanol. The fixed cells were incubated overnight at 4°C with anti-Lyn, -Hck, -Fyn, -Yes, and -c-Src. Fluorescence microscopy was performed after incubation with fluorescein isothiocyanate-conjugated secondary IgG antibodies, followed by 4′,6-diamidino-2-phenylindole staining.

Western Blotting Analysis

hPASMCs were lysed in radioimmunoprecipitation assay lysis buffer (Sigma) containing protease and phosphatase inhibitors (Roche), and the lysates were subjected to Western blots.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was reverse transcribed with ImProm-II Reverse Transcription System, followed by real-time polymerase chain reaction analysis of human and rat Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, c-Src, and porphobilinogen deaminase using the primers described in Table IA and IB in the online-only Data Supplement.

Statistical Analysis

Data are presented as mean±SEM. Unless otherwise stated, statistical comparisons of samples were performed by ANOVA followed by the Dunnett post hoc test. Statistical comparisons for real-time polymerase chain reaction experiments are based on delta cycle threshold values. Samples were compared using Student t test.
Results

Comparison of PDGF-Activated Signaling Inhibition by Imatinib, Nilotinib, and Dasatinib in hPASMCs

To compare the inhibition of PDGF-activated signaling in PASMCs by imatinib, nilotinib, and dasatinib, we incubated serum-starved hPASMCs with different concentrations of inhibitors (imatinib 0.3–3 μmol/L, nilotinib 0.03–0.3 μmol/L, dasatinib 0.003–0.03 μmol/L), followed by stimulation with PDGF-BB for 10 and 30 minutes. PDGF-BB stimulation markedly increased PDGFRβ phosphorylation (p-PDGFRβ), which was detectable in as early as 10 minutes. Furthermore, Western blot analysis of PDGF-activated downstream signaling molecules suggested activation of Akt and signal transducer and activator of transcription 3 (STAT3) by PDGF-BB stimulation (Figure 1A–1C).

As shown in Figure 1A through 1C, 1 to 3 μmol/L imatinib and 0.1 to 0.3 μmol/L nilotinib were required to block p-PDGFRβ. Similarly, at these concentrations, both imatinib and nilotinib markedly inhibited Akt and STAT3 phosphorylation. On the other hand, dasatinib completely blocked PDGF-BB-stimulated p-PDGFRβ at 0.03 μmol/L. Importantly, this concentration of dasatinib also potently inhibited PDGF-induced Akt and STAT3 phosphorylation.

Src Family Members: Expression and Localization in hPASMCs

mRNA analysis of SFKs (Src, Fyn, Yes, Lyn, Fgr, Hck, Lck, and Blk) by real-time reverse-transcription polymerase chain reaction in hPASMCs demonstrated a strong expression of only 5 Src family members, Src, Fyn, Yes, Lyn, and Hck (Figure 2A and 2B). These results were confirmed by immunostaining, which showed the presence of Src, Fyn, Yes, Lyn, and Hck in the cytoplasm (Figure 2B).

Influence of Imatinib, Nilotinib, and Dasatinib on PDGF-Induced Src Tyrosine Kinase Activity in hPASMCs

Because Src activity was shown to be elevated by stimulation of quiescent fibroblasts with PDGF, we studied the time-dependent activation of Src tyrosine kinase activity/phosphorylation by PDGF-BB stimulation in hPASMCs. Accordingly, PDGF-BB markedly increased Src phosphorylation (p-Src) in a time-dependent manner, increasing in as early as 5 minutes, and sustained until 30 minutes (Figure 2C).

Further, we determined whether dasatinib, nilotinib, and imatinib could inhibit PDGF-BB-induced p-Src. Importantly, all 3 inhibitors blocked Src tyrosine phosphorylation in response to PDGF-BB in a concentration-dependent manner (Figure 2D–2F). However, compared with imatinib and nilotinib, maximal reduction of p-Src was achieved even with the lowest concentration of dasatinib (0.03 μmol/L) in PDGF-BB-stimulated PASMCs after 30 minutes (Figure 2F).

Inhibition of PDGF-Induced hPASMC Proliferation by Imatinib, Nilotinib, and Dasatinib

Given the potent inhibitory potential of dasatinib, imatinib, and nilotinib on p-PDGFRβ and PDGF-BB-activated downstream signaling molecules, we studied the effects of each inhibitor on PDGF-BB-induced PASMC proliferation. PDGF-BB stimulation caused an ≈3-fold increase in BrdU uptake compared with nonstimulated cells. As shown in Figure 3A and 3B, imatinib and nilotinib at 3 to 10 μmol/L effectively inhibited PDGF-BB-induced PASMC BrdU uptake. However, dasatinib at 0.03 to 0.1 μmol/L was sufficient to achieve the similar inhibitory effects on PDGF-BB-induced PASMC BrdU uptake, suggesting a 100-fold higher potency of dasatinib (Figure 3C). We further analyzed the effects of dasatinib, imatinib, and nilotinib on PDGF-BB-induced change in cell cycle regulators. Importantly, all 3 inhibitors at the above-mentioned concentrations reversed the PDGF-BB-induced downregulation of the cyclin-dependent kinase inhibitor p27 and induction of cyclin D1. However, there was no effect of PDGF-BB and the above inhibitors on the regulation of other cyclin-dependent kinase inhibitors such as p21 (Figure 3D–3F).

Imatinib, Nilotinib, and Dasatinib Potently Inhibit PDGF-Induced Chemotaxis of hPASMCs

To study the influence of dasatinib, nilotinib, and imatinib on PDGF-BB-induced chemotaxis of hPASMCs, we used a modified Boyden chamber assay. PDGF-BB stimulation increased PASMC chemotaxis ≈3 times the baseline value of nonstimulated cells (Figure 1A–1C in the online-only Data Supplement). Imatinib and nilotinib at 3 to 10 μmol/L, potently inhibited PDGF-BB-induced chemotaxis (Figure 1A and 1B in the online-only Data Supplement). Similarly, dasatinib exerted a strong inhibitory effect on this process. Even at a concentration of 0.03 μmol/L, the PDGF-BB-induced chemotaxis of PASMCs was inhibited by 90% (Figure 1C in the online-only Data Supplement).

Dasatinib But Not Imatinib or Nilotinib Inhibited MGF-Induced Proliferation in hPASMCs

Further considering the putative involvement of several growth factors in the abnormal phenotype of hPASMCs and thus in the pathogenesis of PAH, we studied the inhibition of MGF-activated signaling in hPASMCs by different concentrations of imatinib, nilotinib, and dasatinib. MGF stimulation significantly increased p-PDGFRβ, Akt, and STAT3. Dasatinib reduced this effect in a greater manner compared with imatinib and nilotinib (Figure II A– IIC in the online-only Data Supplement). Therefore, we also compared the antiproliferative potential of imatinib, nilotinib, and dasatinib on MGF-induced hPASMC BrdU uptake. MGF induced an ≈6-fold increase in BrdU uptake compared with nonstimulated cells. Treatment with various concentrations of imatinib and nilotinib did not significantly influence BrdU uptake induced by MGF (Figure 4A and 4B). In contrast, dasatinib effectively inhibited MGF-stimulated hPASMC cellular uptake of BrdU, and a complete inhibition was achieved at 3 μmol/L dasatinib (Figure 4C).

In addition, we studied the effects of imatinib, nilotinib, or dasatinib on MGF-induced hPASMC chemotaxis. Similar to PDGF-BB, MGF increased hPASMC chemotaxis ≈3-fold compared with nonstimulated cells (Figure 4D–4F). All 3 compounds, imatinib, nilotinib, and dasatinib, decreased MGF-induced chemotaxis in a concentration-dependent manner, but dasatinib appeared to be the most effective compound.
Dasatinib But Not Imatinib or Nilotinib Inhibited MGF-Induced p-Src in hPASMCs

Because SFKs interact with, and participate in signaling from, several RTKs in multiple ways, including DNA synthesis, we presumed that the differential effects exerted by imatinib, nilotinib, or dasatinib on MGF-induced PASMC proliferation may involve the regulation of SFKs. Hence, we studied the effects of various growth factors present in our MGF cocktail, namely EGF, basic FGF, and IGF-1, on p-Src after 15 and 30 minutes. Among the 3, IGF-1 and EGF substantially increased p-Src (Figure 5A and 5B), which was abolished by dasatinib (Figure IIIA and IIIB in the online-only Data Supplement). On the
other hand, basic FGF did not show any effect on p-Src (Figure 5C). In agreement with these results, even MGF, consisting of all these growth factors, caused robust p-Src (Figure 5D–5F). Treatment with dasatinib at 0.3 to 3 μmol/L completely abolished this effect. On the contrary, both imatinib and nilotinib had only weak or no influence on MGF-induced p-Src.

To further elucidate the involvement of Src in MGF-induced PASMC proliferation, we used 2 SFK blockers,
phosphoprotein phosphatase 1 (PP1) and PP2. Compared with imatinib treatment alone, cotreatment of imatinib with PP1 or PP2 reduced the MGF-induced PASMC BrdU incorporation (Figure 5G). Similarly, cotreatment of PP1 or PP2 with nilotinib, compared with nilotinib alone, reduced the MGF-induced PASMC BrdU uptake (Figure 5H).

**SFK Activity Is Altered in MCT-Induced PAH PASMCs**

mRNA analysis of SFKs in PASMCs isolated from control and MCT-PH rats by real-time reverse-transcription polymerase chain reaction demonstrated no regulation of SFK expression in MCT-PASMCs (Figure IVA in the online-only Data Supplement). However, we observed an increase in p-Src in MCT-PASMCs compared with control PASMCs (Figure IVB and IVC in the online-only Data Supplement).

**Imatinib, Nilotinib, and Dasatinib Influence Hemodynamics and RV Hypertrophy in MCT-Induced PAH at Different Concentrations**

To assess and compare the therapeutic potential of imatinib, nilotinib, and dasatinib in MCT-PH, rats were treated 3 weeks after MCT injection with the respective RTK inhibitors or placebo for 14 days. Placebo-treated MCT-PH rats exhibited a significant increase in RV systolic pressure on day 35 (83.6 ± 6.4 mm Hg) compared with control rats (29.1 ± 1.4 mm Hg; Figure 6A). Furthermore, compared with control animals (38.5 ± 5.3 mL/min per 100 g bw), the cardiac index was decreased on day 35 (29.6 ± 3.1 mL/min per 100 g bw; Figure 6B). No significant changes in systemic vascular resistance index occurred (Figure VA in the online-only Data Supplement). Importantly, dasatinib (both 5 and 15 mg/kg per bw), nilotinib (30 mg/kg per bw), and imatinib (100 mg/kg per bw) treatment (day 21–35) resulted in a remarkable reduction
of RV systolic pressure to 63.1±4.6, 38.4±2.3, 56.0±5.6, and 47.9±4.4 mm Hg, respectively (Figure 6A). In the dasatinib (15 mg/kg per bw) group, cardiac index significantly increased compared with all other treatment groups (Figure 6B). Systemic arterial pressure increased whereas systemic vascular resistance index did not change in the dasatinib-treated group compared with placebo-treated MCT-PH rats (Figure VA in the online-only Data Supplement). Five weeks after injection of MCT, animals demonstrated significant RV hypertrophy (RV/left ventricle+septum) from 0.22±0.01 (control animals) to 0.57±0.02. All 3 compounds, dasatinib (both concentrations), nilotinib, and imatinib, reduced RV hypertrophy to 0.42±0.02, 0.33±0.01, 0.46±0.03, and 0.40±0.03, respectively (Figure 6C).

Nilotinib and Dasatinib Affect Vascular Remodeling

We quantitatively assessed the degree of muscularization of small pulmonary arteries (diameter 20–70 μm). In controls, the majority of vessels of this size were nonmuscularized (79.5±4.5%), with a small percentage of partially muscularized arteries (14.0±4.0%) and an even smaller number of fully muscularized arteries (6.5±0.5%). In contrast, the percentage of nonmuscularized arteries decreased to 5.7±3.6%, and the percentage of partially and fully muscularized arteries increased to 43.0±3.9% and 51.3±6.1%, respectively, in placebo-treated MCT-PH rat lungs (Figure 6D and 6E). Dasatinib treatment (15 mg/kg per day) led to a remarkable reduction in fully muscularized arteries to 17.5±3.1%, accompanied by a significant increase in partially muscularized arteries to 53.4±5.9% and nonmuscularized pulmonary arteries to 31.3±4.4% (Figure 6D and 6E). In addition, nilotinib treatment also reduced the percentage of fully muscularized arteries compared with placebo-treated MCT-PH rat lungs (Figure 6D and 6E).

Dasatinib Exerts Antiproliferative and Proapoptotic Effects In Vitro and In Vivo

The antiproliferative effects of dasatinib and nilotinib were assessed in vivo by immunostaining for PCNA. In MCT-injected rats, PCNA labeling showed proliferation of vascular cells in distal pulmonary arteries compared with controls (Figure 6E and 6F). Dasatinib and nilotinib treatment significantly reduced the number of PCNA-positive vascular cells compared with placebo-treated MCT-PH rat lungs.

Because dasatinib exerts strong antiremodeling and hemodynamic effects, we examined its effect on apoptosis of PASMCs in vitro (Figure VIA in the online-only Data
Supplement). Treatment with dasatinib, at 3 to 10 μmol/L concentrations, significantly increased apoptosis of PASMCs in the presence and absence of serum. In accordance, dasatinib-treated MCT-PH rat lungs displayed an increased number of terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive apoptotic vascular cells compared with placebo-treated MCT-PH rat lungs (Figure VIB in the online-only Data Supplement).

Imatinib, Nilotinib, and Dasatinib Influence Hemodynamics, RV Hypertrophy, and Vascular Remodeling in Hypoxia-Induced PH

To substantiate our findings obtained in the MCT-PH rat model, another well-established model of PH, namely the hypoxia (HOX)-induced PH mouse model was used. Mice were treated after 3 weeks of HOX with the respective RTK inhibitor or placebo for 14 days along with HOX exposure. Placebo-treated mice showed a significant increase in RV systolic pressure on day 35 (42.4±3.0 mm Hg) compared with control mice (27.4±1.1 mm Hg), which was significantly reduced by dasatinib (15 mg/kg per bw) and imatinib (100 mg/kg per bw) treatment to 37.7±1.6 and 37.6±3.5 mm Hg, respectively (Figure VIIA in the online-only Data Supplement). In contrast, nilotinib (30 mg/kg per bw) did not show a significant decrease in RV systolic pressure. No significant changes in systemic arterial pressure occurred (Figure VIIB in the online-only Data Supplement). The significant increase in RV hypertrophy from 0.23±0.02
(control animals) to 0.35±0.03 (placebo-treated animals) was reduced by all 3 inhibitors dasatinib, nilotinib, and imatinib to 0.30±0.01, 0.31±0.02, and 0.31±0.01, respectively (Figure VIIC in the online-only Data Supplement). In accordance with our findings in MCT-treated rats, dasatinib exerted the most pronounced effect on antagonizing RV hypertrophy (Figure VIIC in the online-only Data Supplement), and dasatinib treatment significantly reduced the number of PCNA-positive vascular cells compared with placebo-treated HOX-PH mouse lungs (Figure VIIID in the online-only Data Supplement). In addition, dasatinib and nilotinib treatment led to a remarkable reduction in partially muscularized arteries, accompanied by a significant increase in nonmuscularized pulmonary arteries compared with the placebo-treated group (Figure VIIE in the online-only Data Supplement).
Discussion

Our data demonstrate that nilotinib and dasatinib are potent inhibitors of PDGF-BB-stimulated cellular responses, i.e., proliferation and migration of PASMCs, comparable with imatinib. On the other hand, dasatinib exerts differential effect on MGF-induced PASMC proliferation compared with imatinib and nilotinib. Using selective inhibitors, we demonstrate that this differential effect of dasatinib is via additional inhibition of SFKs. The crucial role of SFKs in PASMC hyperplasia was further supported by an increased p-Src state in PASMCs isolated from experimental models of PAH. Consistent with these findings, dasatinib treatment reduced vascular cell proliferation in situ and reversed MCT- and HOX-induced PH, and this effect was more pronounced and achieved with a lower concentration compared with nilotinib and imatinib (Figure 6 and Figure VII in the online-only Data Supplement). Taken together, these results suggest that dysregulated SFKs contribute to pulmonary vessel remodeling, and represent an additional therapeutic target in PAH.

Vascular remodeling due to PASMC proliferation and migration is central to the development of PH. PASMC proliferation requires the coordinated interaction of several growth factors, including PDGF, EGF, FGF, and others, that are released in an autocrine/paracrine manner and act as potent mitogens and chemoattractants for PASMCs. In agreement with others, we have found that PDGF is overexpressed and promotes vascular remodeling in PAH. However, inhibition of PDGF-mediated cellular responses by imatinib in PASMCs requires micromolar concentrations. In contrast, near-complete inhibition of PDGFR activation and PDGF-stimulated activities in PASMCs was observed with nanomolar concentration of nilotinib and dasatinib in the present study. Dasatinib and nilotinib effectively blocked PDGF-stimulated PDGFR tyrosine phosphorylation. Furthermore, nilotinib and dasatinib suppressed PDGF-BB-stimulated activation of Akt and STAT3 in PASMCs. Both nilotinib and dasatinib effectively inhibited PDGF-BB-stimulated PASMC migration and proliferation. Direct comparison of imatinib, nilotinib, and dasatinib in PASMCs indicates that nilotinib is ≈10-fold and dasatinib is ≈100-fold more potent than imatinib, suggesting dasatinib is a more potent inhibitor of PDGF signaling than imatinib and nilotinib. This observation is consistent with a recent study by Chen et al., in which dasatinib was shown to be 67-fold more potent than imatinib in the inhibition of PDGFR activation in vascular smooth muscle cells.

Among the other RTKs (EGF, FGF, IGF) that are thought to contribute to the pathobiology of PAH, epidermal growth factor receptor blockade was shown to inhibit PASMC growth, induce apoptosis, and provide partial therapeutic efficacy in animal models of PH. In analogy, increased lung and circulating FGF2 levels have been reported in both experimental and human PAH. Knockdown of FGF2 in pulmonary arterial endothelial cells using small interfering RNA reduced PASMC growth. In addition, IGF-1 was shown to stimulate PASMC proliferation.

Because activation of EGF, FGF, IGF, and insulin receptors was shown to contribute to PASMC proliferative responses, we studied the antiproliferative and antimigratory potential of imatinib, nilotinib, or dasatinib in the presence of MGFs. All 3 inhibitors potently inhibited the MGF-induced migration of PASMCs. In contrary, neither imatinib nor nilotinib affected MGF-induced proliferation of PASMCs. However, dasatinib significantly reduced MGF-induced proliferation of PASMCs, suggesting that in addition to the well-documented role of PDGF, the proliferation of PASMCs is also driven by PDGFR-independent mechanisms. This finding can be explained by the potential of dasatinib to inhibit both PDGFR and SFKs. These data are in line with the studies in other vascular smooth muscle cell types. Dual inhibition of PDGFR and SFKs was shown to inhibit both proliferation and migration in coronary arterial smooth muscle cells, and dasatinib was effective in inhibiting PDGF-stimulated aortic smooth muscle cell migration and proliferation.

The importance of SFKs in the inhibition of abnormal vascular smooth muscle cell activities can be underpinned by the fact that SFKs are the major cytosolic tyrosine kinases in vascular tissue and represent a crucial proximal component of the signaling cascades activated by growth factor receptors. We have observed that PDGF, a key player in the pathogenesis of PAH, potently stimulates Src signaling in PASMCs. Moreover, activation of Src kinases was not only observed in vitro in PASMCs stimulated with MGF, but also in vivo in PASMCs isolated from an experimental model of PH. These findings suggest that SFKs may be a central hub for various growth factors including PDGF, EGF, FGF, and insulin, and suggest that Src activation is necessary for the stimulation of DNA synthesis. This observation is consistent with previous findings, where Src was shown to be necessary and sufficient for human airway smooth muscle cell proliferation and migration.

In this setting, it is noteworthy that treatment with dasatinib, but not imatinib and nilotinib, dose-dependently decreased MGF-induced p-Src/activation. Furthermore, the importance of Src for PASMC proliferation is underlined by the observation that the combination of specific inhibitors of SFKs, PP1 and PP2 with imatinib or nilotinib, efficiently reduced MGF-induced PASMC proliferation that was resistant to single imatinib or nilotinib treatment suggesting the generation of a dasatinib phenotype by this combination approach. Indeed, accumulating evidence has suggested a crucial role of SFKs in pulmonary vascular homeostasis. A direct role of SFKs in the acute contractile response to HOX in small pulmonary arteries of rats has been suggested. On the other hand, activation of c-Src by the serotonin 2B receptor induces p-PDGFRβ and activates cell cycle regulators such as cyclins D and E, thus leading to cell proliferation. Because we have recently shown an upregulation of 5-hydroxytryptamine (serotonin) receptor 2B in PASMCs isolated from patients with PAH, an active involvement of SFKs in PAH pathogenesis can be suggested. Furthermore, the interaction of c-Src with bone morphogenetic protein receptor type II C-terminus and the regulation of this interaction by bone morphogenetic protein ligands strongly implicate SFKs in the pathophysiology of PAH.

Importantly, involvement of Src and PDGFRβ kinases in PASMC proliferation and migration is of direct clinical interest, because dual Src kinase and PDGFRβ inhibitor, dasatinib, was recently approved for the treatment of chronic
myelogenous leukemia resistant to imatinib. In our studies, assessment of the therapeutic potential of dasatinib (5 and 15 mg/kg bw) and nilotinib (30 mg/kg bw) in vivo demonstrated reversal of established MCT- and HOX-induced PH in rats and mice. Beneficial effects were observed in 2 different animal models of PH that address media hypertrophy along with the aspect of interspecies differences. For instance, studies have clearly demonstrated interspecies differences in the responses to stimuli capable of promoting PH. Nevertheless, it would be interesting to carry out similar studies in 2 additional models, namely pneumonectomy-MCT and Sugen-HOX, characterized by neonintima formation. Importantly, head-to-head comparison of imatinib, nilotinib, and dasatinib demonstrated a stronger reverse remodeling potential of dasatinib compared with nilotinib and imatinib. The more pronounced antihypertensive effects of dasatinib achieved even with lower concentrations of the inhibitor are most likely explained by its additional ability to inhibit MGF-induced Src activation. Similar preclinical efficacy of dasatinib has also been demonstrated in dermal fibrosis and systemic sclerosis. To our knowledge, this is the first study to describe the reverse remodeling potential of dasatinib in PH.

Despite our finding that dasatinib was more efficient in reducing pulmonary vascular remodeling and experimental PH than other tyrosine kinase inhibitors, current observations indicate that chronic treatment with dasatinib for chronic myeloid leukemia was associated with the onset of severe PAH in humans. In fact, the association is strong enough that the Food and Drug Administration has relabeled dasatinib with a specific warning about the risk of PAH with its use. This effect, however, appears to be mechanistically different, because PAH occurred in conjunction with pleural effusions and appeared to be reversible after termination of dasatinib. Therefore, it is assumed that the pathophysiological mechanism leading to an increase of pulmonary vascular resistance by dasatinib in humans differs from the vascular alterations typically seen in PAH. These effects appear to be functional whereas the dasatinib properties described in the present study are due to antiproliferative effects. Although the mechanism of PAH induction in humans remains elusive, it is likely to be related to off-kinase inhibition which may affect pulmonary vascular wall integrity, possibly leading to (peri)vascular edema and subsequent pulmonary vascular resistance increase. Our data indicate that the additional effect of dasatinib on experimental PH is due to the additional targeting of SFKs by this compound, because the addition of Src inhibitors to imatinib was able to mimic the full dasatinib effect in vitro. Although our data are intriguing, it must be emphasized that carefully controlled clinical trials are absolutely necessary before extrapolating the animal model studies to the complex pathophysiology of human PAH. Effective, approved therapies for PAH exist, and especially because dasatinib has a Food and Drug Administration–labeled warning regarding the possibility for PAH, compassionate use or similar off-label use of dasatinib is inappropriate.

In summary, our study explores the regulation and previously undefined role of SFKs in the pathogenesis of PAH. The present study suggests that a dual inhibitor of PDGFR and SFKs potently interferes with motogenic and mitogenic responses of PASMCs in vitro and in vivo and thus, may represent a novel therapeutic approach to the treatment of PAH.

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Disclosures

None.

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Role of Src Tyrosine Kinases in Experimental Pulmonary Hypertension
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Supplement Material

Role of Src tyrosine kinases in experimental pulmonary hypertension

Pullamsetti, Src’s in pulmonary hypertension

Material and methods

Animal experiments

The experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals. Both the University Animal Care Committee and the Federal Authorities for Animal Research of the Regierungspräsidium Giessen (Hessen, Germany) approved the study protocol.

Experimental groups

Adult male Sprague-Dawley rats (300–350 g in body weight; Charles River Laboratories) were randomized for treatment 21 days after a s.c. injection of saline or 60 mg/kg MCT (Sigma-Aldrich) to induce pulmonary hypertension. In addition to a group of untreated rats, the experimental groups included rats that received once-daily oral BMS-354825 (Dasatinib) at a dose of 5 and 15 mg/kg, AMN10 (Nilotinib) at a dose of 30mg/kg, imatinib at a dose of 100mg/kg or the placebo (2% methocell) via gavage. Rats were examined after 14 days of treatment (on day 35).

Surgical preparation, measurement of hemodynamics, and tissue preparation

To monitor hemodynamics, the animals were initially anesthetized intraperitoneally with ketamine and xylazine. The left carotid artery was cannulated, and a right heart catheter
was inserted through the right jugular vein for measurement of RV pressure with fluid filled force transducers. Cardiac output was measured by a thermodilution technique (Cardiotherm 500-X; Hugo-Sachs Electronic—Harvard Apparatus GmbH, March, Germany) as described\(^1\). After exsanguination, the left lung was fixed for histology in 10% neutral buffered formalin, and the right lung was snap-frozen in liquid nitrogen for molecular biology experiments.

**Assessment of RV hypertrophy**

The RV wall was separated from the left ventricular (LV) wall and ventricular septum. Dry weight of the RV wall, free LV wall, and ventricular septum was determined. RV hypertrophy was expressed as the ratio of weight of the RV wall to that of the free LV wall and ventricular septum (S) [RV/(LV+S)].

**Pulmonary vascular morphometry**

The formalin-fixed lungs were subject to paraffin embedding. The paraffin-embedded tissues were subject to sectioning to yield 3 µm thick sections. The degree of muscularization of small peripheral pulmonary arteries was assessed by double-staining the 3 µm sections with an anti-\(\alpha\)-smooth muscle actin antibody (dilution 1:900, clone 1A4, Sigma, Saint Louis, Missouri) and antihuman von Willebrand factor antibody (vWF, dilution 1:900, Dako, Hamburg, Germany) followed by analysis of the vessels using a computerized morphometric analysis system (QWin; Leica, Wetzlar, Germany) to determine the degree of pulmonary artery muscularization. In each rat, 80 to 100 intra-acinar arteries (25 to 50 µm diameter) were categorized as muscular, partially muscular, or non-muscular.
Assessment of vascular proliferation in vivo

Tissue sections were stained for proliferating cell nuclear antigen (PCNA) using rabbit anti-PCNA (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and visualized with corresponding secondary antibody conjugated with alkaline phosphatase. Color development was performed with a substrate/chromogen mixture followed by counterstaining with hematoxylin. The sections were examined under a Leica DM 2500 microscope using Leica QWin imaging software (Leica). PCNA positive pulmonary vascular cells were counted throughout the entire section and expressed as fold change in percentage of the control lung by calculating the number of PCNA-positive cells per pulmonary vessel.

Pulmonary arterial smooth muscle cell isolation and culture

Primary culture of human pulmonary arterial smooth muscle cells (hPASMCs) was obtained from Lonza (Basel, Switzerland) and grown in SmGM-2 Bulletkit medium (Lonza). Cells were maintained at 37°C in a humidified 95% air and 5% CO2 incubator. Experiments were performed with cells from passages 4-7. Rat PASMCs were cultured from peripheral small pulmonary artery explants as previously described. Small pulmonary arteries were freshly obtained from rats and maintained in Hank's balanced salt solution (HBSS, Gibco) supplemented with penicillin (100 units/ml, PAN) and streptomycin (100 units/ml, PAN). Under a dissecting microscope, the adventitia layer was removed. Arterial segments were cut open along the longitudinal axis, and the endothelium was gently removed by scraping the luminal surface. The arteries were minced into small pieces and maintained in Dulbecco's...
modified Eagle's medium/F12 (DMEM/F12, Gibco) supplemented with 20% fetal bovine serum (FBS, Biowest), penicillin (100 units/ml), streptomycin (100 units/ml), and 2 mM L-glutamine (PAN). After 7 days, PASMCs started to migrate from the explants, and this was followed by 10 days of culturing. PASMCs from passages 2–5 were used for all experiments. Cells were positively stained for α-smooth muscle actin by immunocytochemistry. Every experiment was performed with primary PASMCs isolated from at least 3 individual rats.

**DNA synthesis assay**

DNA synthesis was measured by a 5-bromo-deoxyuridine (BrdU)-incorporation assay as described. Briefly, cells were cultured in 96-well-plates to 90% confluence, washed, fed with DMEM and starved for 24 hr. PDGF-BB (30 ng/ml) or MGF (PDGF-BB, 30 ng/ml; FGF, 2 ng/ml; EGF, 0.5 mg/ml; Insulin, 0.5 μg/ml, FCS, 5%) was added to cells for 18 hr at the indicated concentrations in the absence or presence of various concentrations of Imatinib, Dasatinib or Nilotinib. The BrdU- incorporation assay was carried out according to the manufacturer’s specifications (Roche Diagnostics GmbH, Mannheim, Germany) with an incorporation time of 5 hr.

**Chemotaxis assay**

PDGF- or MGF- dependent chemotaxis was assayed utilizing a 48- well modified Boyden chemotaxis chamber (NeuroProbe Inc., Baltimore, MD) and PVP-free polycarbonate filters (8μm pore size) (Poretics Corp., Livermore, CA) as described previously. Briefly, the lower wells of the chamber were filled with DMEM
supplemented with PDGF-BB (30 ng/ml) or MGF (PDGF-BB, 30 ng/ml; FGF, 2 ng/ml; EGF, 0.5 mg/ml; Insulin, 0.5 µg/ml, FCS, 5%) in the presence or absence of Imatinib, Dasatinib or Nilotinib. The filters were coated with rat type I collagen (50 mg/ml, Collaborative Biomedical Products, Bedford, MA) and fixed atop the bottom wells. PASMCs were trypsinized, washed and diluted in DMEM to a final concentration of 4x10^5 cells per ml. 50 µl of this cell suspension was placed into the top wells. In each experiment, at least 6 of the chamber’s 48 wells were used for each condition examined. The chamber was incubated for 4 hr at 37° C in a 5% CO₂ atmosphere. Following incubation, the chamber was disassembled, the cells on the upper surface of the filter were removed, and the cells on the lower surface fixed and stained with Diff-Quick (Baxter Healthcare Corp., Miami, FL). Chemotaxis was quantified by counting the number of cells on the lower surface of the filter in each well using a grid containing 100 non-overlapping fields. The total number of cells per 100 fields was 5–20 in resting cells, and 80–150 in responding cells. The response being measured was primarily chemotaxis, since including PDGF in the top and bottom chamber reduced the number of migrated cells by approximately 70%.

**Immunofluorescence staining of pulmonary arterial smooth muscle cells**

Immunofluorescence labeling of human PASMCs was performed as described. Antibodies directed against Lyn (1:100, Abcam), Hck (1:100, Santa cruz), Fyn (1:100, BD Biosciences), Yes (1:100, BD Biosciences), and c-Src (1:100; Cell signaling) were used and visualized with Alexa 488- conjugated goat anti-rabbit IgG, Alexa 488-
conjugated donkey anti-goat IgG, Alexa 488- and conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, MA). At the end of the procedure, the slides were examined by fluorescence microscopy after DAPI staining.

**Western blotting**

Proteins extracted from PASMCs with RIPA buffer (Santa Cruz) were resolved with SDS-PAGE (7% and 10% acrylamide gels) and transferred onto nitrocellulose membranes. After being blocked with 5% non-fat milk or BSA for 1 h at room temperature, membranes were probed with primary antibodies overnight at 4°C as follows: rabbit anti-PDGFRβ antibody (1:200), rabbit anti-phospho-PDGFRβ (Tyr1021) antibody (1:200), mouse anti-p27 (1:200), rabbit anti-cyclinD1 (1:200); all from Santa Cruz Biotechnology Inc (Santa Cruz, CA); rabbit anti-Akt (1:1000), rabbit anti-phospho-Akt (Ser473) antibody (1:1000), rabbit anti-Stat3 antibody(1:1000), rabbit anti-phospho-stat3 (Ser727) antibody (1:1000), rabbit anti-src (1:1000) antibody, rabbit anti-phospho-src family (Tyr416) antibody (1:1000) all from Cell Signaling Technology (Beverley, MA); mouse anti-p21 antibody (1:1000) from BD Biosciences (San Jose, CA); mouse anti-GAPDH antibody (1:5000) from Novus Biological (Littleton, CO, USA). Following washing with TBS containing 0.1% Tween20, HRP-conjugated secondary antibodies (1:40000; anti-rabbit, Pierce; 1:50000 anti-mouse, Sigma-Aldrich) were applied for 1 h. After washing, the blots were developed with an enhanced chemiluminescence (ECL) kit (Amersham Bioscience, Freiburg, Germany) followed by film exposure and densitometric quantification.
RNA isolation, cDNA synthesis, and relative mRNA quantification by real-time reverse-transcription PCR

PASMCs were trypsinised and RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. For cDNA synthesis, total RNA was reverse transcribed using the ImProm-II™ Reverse Transcription System (Promega, Mannheim, Germany). Aliquots were used for real-time [reverse transcription (RT)] polymerase chain reactions (PCRs) using the CFX96 Real Time System (BioRad, Muenchen, Germany) and SYBR Green (iQ SYBR Green supermix, BioRad, Muenchen, Germany) as the fluorescence signal. The expression of human and rat Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, and c-Src was normalized to the housekeeping gene porphobilinogen deaminase (PBGD) and expressed as DCT. The primers used in this study are shown in Table 1A and 1B.

In vitro and in vivo apoptosis assay

For in vitro studies, hPASMCs were grown on chamber slides, serum starved for 24hrs and treated as indicated (3 and 10µM dastinib) in presence or absence of serum,. After 24 hours, cells were fixed immediately in 4% (vol/vol) paraformaldehyde for 1 hour, permeabilized using Triton X-100 (Sigma-Aldrich), For in vivo apoptosis studies, paraffin sections from MCT and dasatinib treated rats, were deparaffinised, rehydrated and permeabilised with proteinase K. solution. Then, both chamber slides and tissue sections were incubated at 37°C for 60 minutes with TdT-mediated TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics Corp.). For
positive control, cells or sections were treated with DNase I (Roche Diagnostics Corp.) as specified by the manufacturer.

References


# Table IA: Human Primer sequences

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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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# Table IB: Rat Primer Sequences

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Supp Figure. III

(a) Western blot analysis of pSrc, Src, and GAPDH with increasing concentrations of IGF (30ng/ml) and Dasatinib (μM).

(b) Western blot analysis of pSrc, Src, and GAPDH with increasing concentrations of EGF (30ng/ml) and Dasatinib (μM).

(c) Graph showing the expression ratio of pSRC/SRC in the presence of IGF and Dasatinib.

(d) Graph showing the expression ratio of pSRC/SRC in the presence of EGF and Dasatinib.
Supp. Figure IV

(a) 

(b) 

(c)
Supp. Figure VI

(a) Bar graph showing the fold increase in apoptosis with different growth medium and Dasatinib concentrations. 

(b) Immunofluorescence images comparing control, MCT (35d), and Dasatinib (15mg) treatments.
Figure legends

**Supp. Figure I: Effects of imatinib, nilotinib or dasatinib on PDGF-induced chemotaxis in human PASMCs**

(a-c) hPASMCs were stimulated with PDGF-BB in the presence of various concentrations of imatinib, nilotinib, dasatinib or DMSO (-) and measured for the changes in chemotaxis utilizing modified Boyden chambers. All data are expressed as the percentage of the PDGF response and represent means ± SEM, n=4. *, p<0.05 versus non-stimulated; †, p<0.05 versus PDGF- treated group.

**Supp. Figure II: Effect of imatinib, nilotinib or dasatinib on MGF-stimulated PDGFR-, Akt- and Stat3- phosphorylation in human PASMCs**

(a-c) hPASMCs were preincubated with indicated concentrations of imatinib, nilotinib, dasatinib or DMSO (-) and then treated with MGF. Cell lysates were analyzed by western blotting with antibodies to p-PDGFRβ, PDGFRβ, p-Akt, Akt, p-Stat3, Stat3 and GAPDH. n=4.

**Supp. Figure III: Effect of dasatinib on EGF- and IGF-stimulated Src phosphorylation in human PASMCs**

hPASMCs were preincubated with dasatinib or DMSO (-) and then treated with IGF (a) and EGF (b). Cell lysates were analyzed by western blotting with antibodies to pSrc, Src and GAPDH and (c, d) densitometric quantification of p-Src to Src in PASMCs n=2.
**Supp. Figure IV: Src family kinases expression and activity in monocrotaline (MCT)-induced PAH rats**

(a) mRNA expression of Src family kinases in PASMCs isolated from control and monocrotaline (MCT)-induced PAH as analyzed by real-time polymerase chain reaction. All values were normalized to porphobilinogen deaminase (PBGD), and relative changes were expressed as ΔCT. n=3. (b) Western blotting with antibodies to p-Src (Y416), Src, GAPDH and (c) densitometric quantification of p-Src to Src in PASMCs isolated from control and MCT-induced PAH. All values are means ± SEM. n=3.

**Supp. Figure V: Influence of imatinib, nilotinib or dasatinib on hemodynamics in monocrotaline (MCT)-induced PAH**

Physiological measurements were taken 35 days after MCT injection. Imatinib (100mg/kg, n=8), dasatinib (5mg/kg (n=9) or 15mg/kg (n=8)), nilotinib (30mg/kg, n=7) or placebo (n=11) was applied orally from day 21 to day 35. (a) systemic vascular resistance index (SVRI) and (b) systemic arterial pressure (SAP). All values are given as mean ± SEM. *, p < 0.05 versus control; †, p < 0.05 versus MCT (5W). (c) Western blotting with antibodies to p-Src (Y416), Src, GAPDH from dasatinib treated rat lung homogenates. All values are means ± SEM. n=4.

**Supp Figure VI: In vitro and in vivo pro-apoptotic effects of dasatinib**

(a) hPASMCs were stimulated with or without growth medium in the presence of dasatinib (3µM, 10µM) or DMSO (-) and measured for the apoptosis. All data are expressed as the fold increase of DMSO (-) response and represent means ± SEM, n=4. *,
p<0.05 versus without or with growth medium treated group. (b) Representative images of placebo and dasatinib treated MCT-PH rat lung sections that were assessed for apoptosis using in situ cell death detection method (TUNEL assay). Arrows indicate TUNEL positive cells; scale bars = 20 µm.

Supp. Figure VII: Influence of imatinib, nilotinib or dasatinib on hemodynamics and pulmonary vascular remodeling in hypoxia (Hox)-induced PH

Physiological measurements were taken after 35 days of hypoxia exposure. Imatinib (100mg/kg, n=6), dasatinib (15mg/kg (n=7)), nilotinib (30mg/kg, n=7) or placebo (n=8) was applied orally from day 21 to day 35. (a) Right ventricular systolic pressure (RVSP), (b) measurement of RV hypertrophy [RV/(LV+S)], (c) systemic arterial pressure (SAP), (d) degree of muscularization and (e) PCNA-positive vascular cells were counted and expressed as index of proliferation (IOP). All values are given as mean ± SEM. *, p < 0.05 versus control; †, p < 0.05 versus Hox (35 days).