Cyclin-Dependent Kinase Inhibitor $p27^{Kip1}$, But Not $p21^{WAF1/Cip1}$, Is Required for Inhibition of Hypoxia-Induced Pulmonary Hypertension and Remodeling by Heparin in Mice

Lunyin Yu, Deborah A. Quinn, Hari G. Garg, Charles A. Hales

Abstract—Heparin has growth inhibitory effects on pulmonary artery smooth muscle cell (PASMC) in vitro and in vivo. However, the mechanism has not been fully defined. In this study, we investigated the role of cyclin-dependent kinase inhibitors, $p21^{WAF1/cip1}$ (p21) and $p27^{Kip1}$ (p27), in the inhibitory effect of heparin on PASMC proliferation in vitro and on hypoxia-induced pulmonary hypertension in vivo using p21 and p27-null mice. In vitro, loss of the p27 gene negated the inhibitory effect of heparin on PASMC proliferation, but p21 was not critical for this inhibition. In vivo, heparin significantly inhibited the development of hypoxia-induced pulmonary hypertension and remodeling, as evidenced by decreased right ventricular systolic pressure, ratio of right ventricular weight to left ventricle plus septum weight, and percent wall thickness of pulmonary artery, in $p21^{+/+}$, $p21^{-/-}$, $p27^{+/+}$, and $p27^{+/-}$, but not in $p27^{-/-}$ mice. We also observed that hypoxia decreased p27 expression significantly in mouse lung, which was restored by heparin. Heparin inhibited Ki67 proliferative index in terminal bronchial vessel walls in $p27^{+/+}$ and $p27^{+/-}$, but not in $p27^{-/-}$ mice exposed to hypoxia. Therefore, we conclude that the cyclin-dependent kinase inhibitor p27, but not p21, is required for the inhibition of hypoxic pulmonary vascular remodeling by heparin. (***Circ Res*. 2005;97:937-945.)**

Key Words: $p27^{Kip1}$ ■ $p21^{WAF1/cip1}$ ■ heparin ■ pulmonary hypertension ■ hypoxia ■ mouse

Heparin, a glycosaminoglycan, has been used as an anticoagulant for more than 50 years.[1] Besides anticoagulation, heparin has a variety of other biological activities, such as regulation of lipid metabolism, control of cell attachment to various proteins in the extracellular matrix, binding with acid and basic fibroblast growth factors, and inhibition of vascular smooth muscle cell (SMC) proliferation.[2]

An important pathological feature of pulmonary hypertension is increased medial thickening of the pulmonary artery attributable to hypertrophy and hyperplasia of pulmonary artery SMC (PASMC).[3,4] Our previous studies have shown that antiproliferative heparins significantly inhibit pulmonary vascular remodeling induced by hypoxia in rodents[5–7] and PASMC proliferation in culture.[8–10] Other investigators also have reported that heparin inhibits PASMC proliferation in vitro and in vivo.[8,11] To date, however, the mechanism by which heparin inhibits PASMC proliferation has not been elucidated.

The balance between cell proliferation and cell quiescence is regulated by a variety of cell cycle modulators. Cyclin-dependent kinase (CDK) is a major regulator of the transition between the phases of the cell cycle.[12] Cyclin/CDK complexes are composed of a regulatory subunit, cyclin, and an active kinase subunit, CDK. The cyclin/CDK complexes are controlled by both positive and negative regulators.[13] $p21^{WAF1/cip1}$ (p21) and $p27^{Kip1}$ (p27) are two primary negative regulators of CDK in SMC and play an important role in the inhibition of CDK activity.[14] Both p21 and p27 inhibit the phosphorylation of cyclin A/CDK2, cyclin D/CDK4, and cyclin E/CDK2 complex, which results in inhibition of the activity of this complex and cell growth arrest in G_1 phase.[12]

Fouty et al observed that overexpression of p27 decreased PASMC proliferation.[15] Other investigators have found that overexpression of p27 was associated with attenuated systemic artery SMC proliferation.[16,17] The first identified negative regulator of CDK, p21, has also been reported to have inhibitory effects on artery smooth muscle cell proliferation.[14] Many studies have found that inhibition of SMC proliferation was accompanied by upregulation of p21 activity.[17–18] Khoury and Langleben[11] reported an increase in p21 with heparin inhibition of pericyte proliferation although the role of p21 was not defined.

Based on our previous findings and other investigators' observations, we hypothesized that p21 and p27 play an important role in the inhibition of PASMC proliferation and of hypoxia-induced pulmonary hypertension by heparin. Therefore, the objective of this study was to investigate the

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role of the CDK inhibitors p21 and p27 in the inhibitory effect of heparin on PASMC proliferation in vitro and in the development of hypoxia-induced pulmonary hypertension and remodeling in vivo.

Materials and Methods

Cell Culture and Treatment

Bovine PASMCs were isolated from bovine pulmonary arteries as previously described.[19] $p27^{-/-}$ and wild-type control ($p27^{+/+}$) mouse PASMCs from pulmonary arteries of p27-null and C57 BL/6 mice were provided kindly by Brian W. Fouty at University of Colorado Health Science Center, Denver, Colorado. The cells, grown in RPMI medium1640 with 10% FBS, streptomycin, penicillin, and amphotericin B, were used in passages 4 through 6. Cell growth assays were performed on PASMC in passage 4 (n=5), passage 5 (n=5), and passage 6 (n=5) to ensure reproducibility. The cells were seeded at 1.25×10^4 cells per well in 6-well tissue culture plates, were allowed to grow for 2 days, and were growth arrested for 48 hours. The media was then changed either to standard medium (with 10% FBS), to growth arrest medium (with 0.1% FBS), or to standard medium with heparin at different doses. Upjohn heparin (batch #1209b) from beef lung was a gift from Pharmacia & Upjohn Inc, Kalamazoo, Mich., and was used for cell cultures and animal experiments in this study. After treatment with heparin for 4 days, the cells were harvested for cell proliferation assays using a direct cell-counting proliferation assay[9,10] and for Western blot and RT-PCR analysis. The percent growth was calculated as (net cell growth in treated medium/net cell growth in standard medium)×100, where the net cell growth=cell growth in standard or treated medium−cell growth in growth arrest medium.[9,10]

Western Blot

Total cell lysates were obtained from harvested cells. Antibodies included p21 rabbit polyclonal antibody (C-19; Santa Cruz Biotechnology, Santa Cruz, Calif), p27 mouse monoclonal antibody (clone 57; BD Biosciences Pharmingen, San Diego, Calif), and GAPDH mouse monoclonal antibody (clone 6C5; Research Diagnostics, Inc, Flanders, N.J.).

RT-PCR

Total RNA was extracted from cultured PASMCs. Total RNA (4 μg) was used to carry out RT-PCR to measure mRNA expression with Qiagen Onestep RT-PCR Kit (Qiagen Inc). The primer pairs for p27,[20] for p21,[21] and for the housekeeping gene GAPDH[22] were purchased from Sigma Genosys, Woodlands, Tex.

siRNA In Vitro Gene Silencing

In vitro siRNA transfections were performed using a Qiagen RNAi starter Kit (Qiagen, Inc). After treatment with heparin for 4 days, the cells were harvested for cell growth assay. Western blot analysis was performed to confirm the gene silencing by small interfering RNA (siRNA). According to manufacturer's directions, p27 siRNA was designed on the basis of the p27 gene sequence (GeneBank accession no. NM 004064-2) at Qiagen's website, siRNA Design tool by Sequence. The DNA target sequence for this p27 gene was AAGGTGCATACTGAGCCAAG, and the siRNA duplex sequences were sense 5′-GGUUGCAUACUGAGCCAAG-3′ and antisense 3′-TCCAACGUAUGACUCGGUUC-5′. p21 siRNA was designed according to previously published work by Zou et al.[23] p27 siRNA and p21siRNA were synthesized by Qiagen-Xeragon, Inc.

Animals

Animal experiments were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. A total of 116 mice were used. Homozygous p21-null male mice ($p21^{-/-}$, p21-knockout[KO]) were the gifts of Dr Philip Leder (Department of Genetics, Harvard Medical School, Boston, Mass), and the strain-specific FVB wild-type (WT) control mice ($p21^{+/+}$) were obtained from Taconic Farmer, Inc (Germantown, NY). Homozygous p27-null ($p27^{-/-}$, p27-KO) and heterozygous (Het) p27 ($p27^{+/-}$, p27-Het) male mice and the strain-specific129S4 control mice ($p27^{+/+}$, WT) were bred from a breeding pair of Het p27 mice ($^{+/-}$) (gifts from Dr Jim Roberts, Fred Hutchinson Cancer Research Center, Seattle, Wash), and the offspring genotype was confirmed by polymerase chain reaction of genomic DNA.[24] The mice (8 to 10 weeks old) were placed in a hypoxic chamber or exposed to normoxia in the same chamber for 2 weeks. Oxygen concentration was maintained at 10% by controlling the flow rates of compressed air and N_2. Cage concentration of O_2 was checked daily. The heparin-treated mice were given 300 U/kg of heparin subcutaneously twice daily for 14 days, as in our previous study.[5] In control groups, mice were given 0.1 mL of saline subcutaneously twice daily.

Measurements of Right Ventricular Pressure

After 14 days in the chamber, the animals were removed and anesthetized with intraperitoneal ketamine (80 mg/kg) and diazepam (5 mg/kg). Animals were placed on a warming blanket to maintain body temperature at 37°C. Right ventricular systolic pressure (RVSP) was measured with the use of a single lumen catheter (0.012×0.016 inches silicone tubing) passed through the right external jugular vein. The animals were then euthanized with 200 mg/kg of pentobarbital and used immediately for the determination of right ventricular hypertrophy, hematocrit, and lung pathology as well as gene expression.

Histological Evaluation

Right ventricular hypertrophy was measured as the ratio of right ventricular weight to left ventricular plus septal weight (RV/LV+S). Pulmonary vascular remodeling was assessed by measuring the percentage of wall thickness of the vessels (%WT), including terminal bronchial and intraacinous arterioles. The percentage of thick-walled as a fraction of total intraacinous vessels (% thick) was also determined.[5,25] A computer imaging analysis was applied for the measurement of wall thickness. The images of individual pulmonary arteries were captured using a digital camera, mounted on a light microscope, and linked to a computer. Wall thickness was measured as described previously.[5,25]

Detection of the CDK Inhibitors

Total RNA and protein were isolated from the mouse lungs and the same methods were used for the detection of the expression of p27 and p21 mRNA and protein as described above.

Immunohistochemical Staining for Ki67 Expression

Anti-Ki67 antibody (rabbit polyclonal, dilution 1:25; Abcam, Inc, Cambridge, Mass) was used as a marker of vascular wall cell proliferation. Immunohistochemical staining of paraffin sections of lung tissue was performed by using a labeled-(strept)avidin-biotin (LAB-SA) detection kit (Histostain-plus kit; Zymed Laboratory, Inc) following the manufacturer's protocol. Hematoxylin was used as counterstain. Control slides were treated identically but without the primary antibody. The identification of cellular positive status was determined by Ki67 nuclear staining by a blinded investigator. The percentage of Ki67 positive cells was estimated by calculating the ratio of Ki67-expressing cell nuclei to the total number of cell nuclei in the cell wall of cross-sections of 10 terminal bronchial arterioles per slide.

Hematocrit Measurement

Blood samples were collected and centrifuged in heparinized microcapillary tubes for 3 minutes. Hematocrit was read directly.

Statistical Analysis

All values were expressed as mean±SEM. Statistics were performed using the computer program Statview (SAS Institute, Inc) with

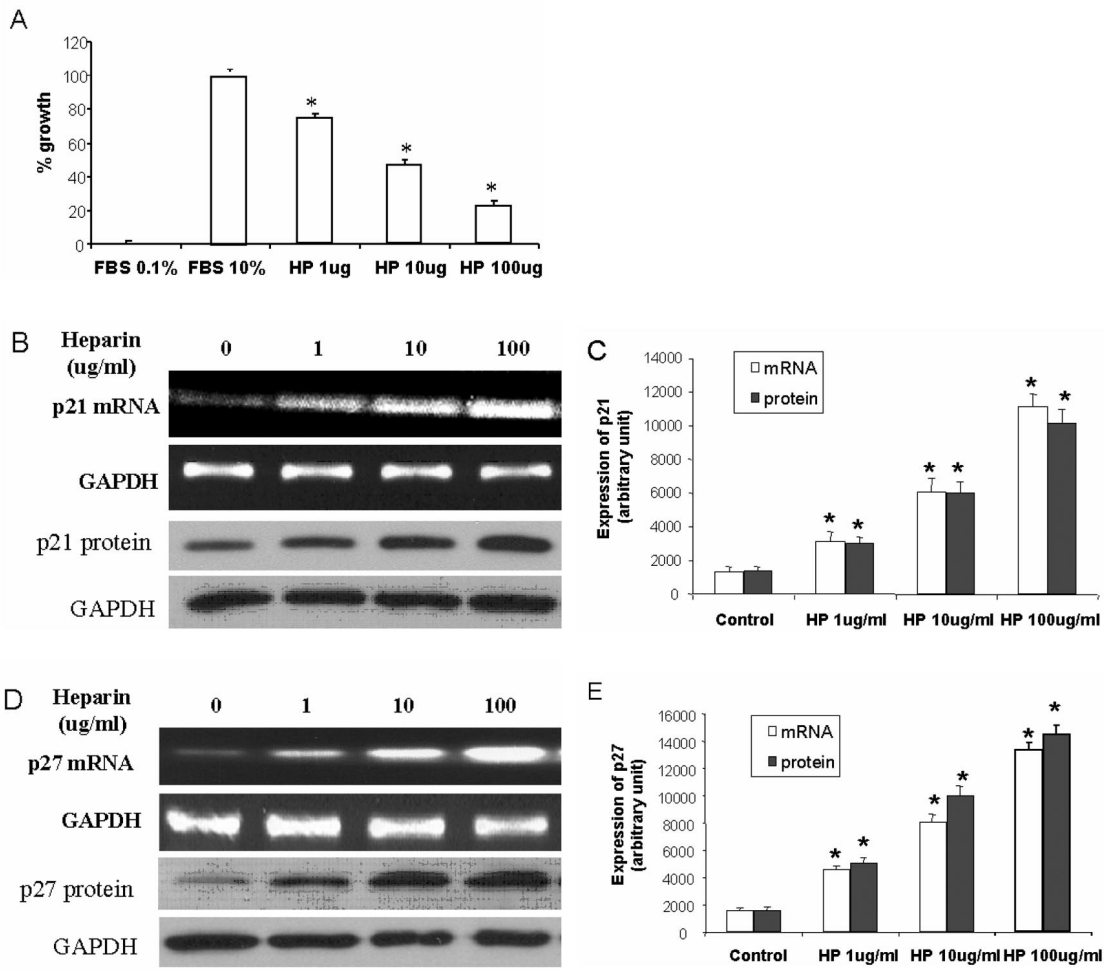


Figure 1. A, Cell growth assay. Bovine PASMC were incubated for 4 days with heparin in the medium, and then cell growth assay was performed. Cells grown in 10% FBS and 0.1% FBS were used for positive and negative controls. The results were representative of 3 separate experiments (total n=15), and error bars represent standard error. *$P<0.05$ as compared with 10% FBS. HP indicates heparin. B through E, Detection of p21 and p27 mRNA and protein expression. Total RNA and protein from bovine PASMC grown for 4 days in medium containing 10% FBS and the indicated amount of heparin were isolated and then subjected to RT-PCR and Western blot analysis. GAPDH was used for equal loading control. Controls represent bovine PASMC grown in 10% FBS without added heparin. Expression of p21 and p27 mRNA and protein, which were representative of 5 separate experiments in B and D, were quantified as arbitrary unit (AU) of densitometry of the band images (C and E) from RT-PCR for mRNA expression and from Western blot for protein expression by using National Institutes of Health 1.61 image software. *$P<0.05$ as compared with control.

factorial ANOVA. If ANOVA were significant, multiple comparisons were made among groups using the Fisher protected least significant difference test. Significance was set at $P<0.05$.

Results

Heparin Induced PASMC Growth Arrest and Increased p21 and p27 mRNA and Protein Expression

Bovine PASMCs stimulated with 10% FBS were treated with heparin for 4 days, which significantly inhibited PASMC growth in a dose-dependent manner compared with nonheparinized controls ($P<0.05$ versus 10% FBS; Figure 1A), and which caused a dose-related increase in p21 mRNA and protein ($P<0.05$ versus control; Figure 1B and 1C) as well as in p27 mRNA and protein ($P<0.05$ versus control; Figure 1D and 1E).

Blockade of the p21 Gene Did Not Affect the Inhibitory Effect of Heparin on PASMC Proliferation

PASMC growth was inhibited significantly and in a dose-dependent manner by heparin despite 70% inhibition of p21 protein expression by p21 siRNA transfection ($P<0.05$ versus 10% FBS; Figure 2A and 2B). These data suggested that the p21 gene was not critical in heparin-induced inhibition of PASMC proliferation. To examine the role of heparin in the complete absence of p21, we observed that heparin significantly inhibited the 10% serum-induced proliferation of both HCT 116 $p21^{+/+}$ and $p21^{-/-}$ colon cancer cells. The percent growth was 63% and 42% in $p21^{+/+}$ cells and 75% and 51% in $p21^{-/-}$ cells, respectively, at doses of 100 μg/mL and 200 μg/mL of heparin, compared with 100% growth in serum without heparin. Thus, these cells showed strong inhibition by heparin even in the absence of p21.

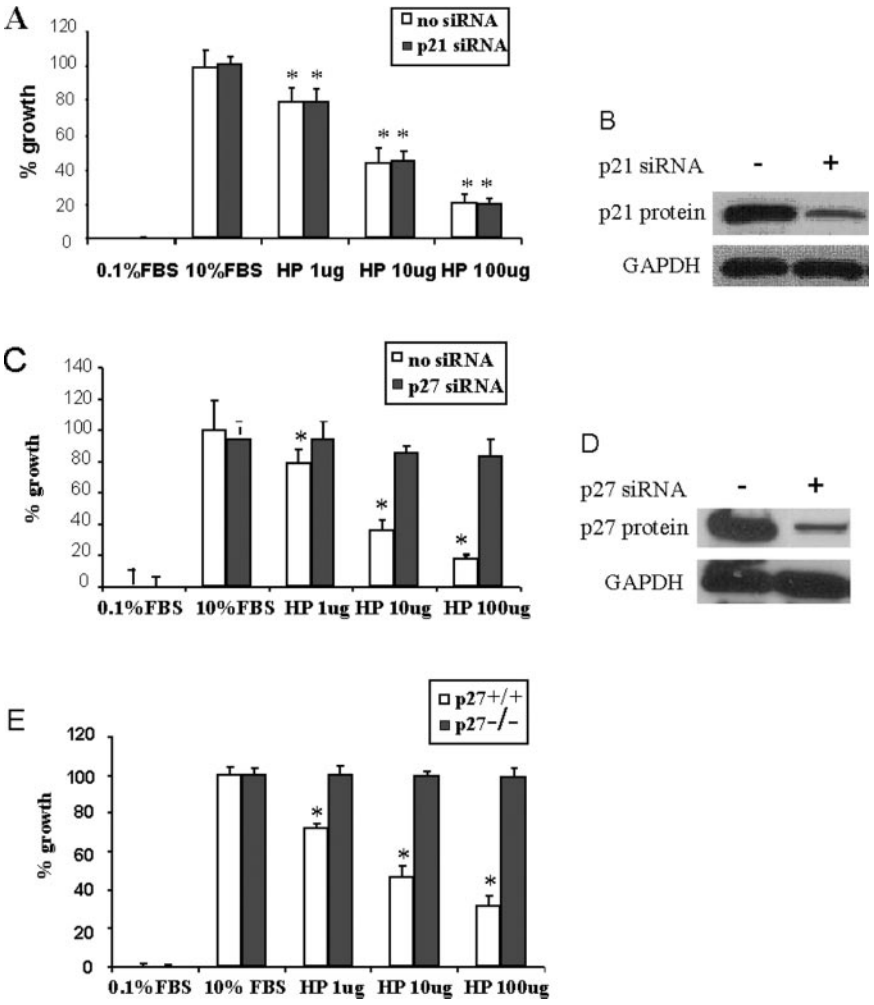


Figure 2. A through D, p21 and p27 siRNA silencing and PASMC proliferation. Bovine PASMC grown to 50% confluence were transfected with siRNA of both CDK inhibitors at dose of 1.5 μg per well in 24-well plates for 24 hours. After serum-starvation for 48 hours, the cells transfected with siRNA were incubated for 4 days with heparin. Cells grown in 10% FBS or 0.1% FBS were used for positive and negative control. A, % growth of the cells transfected with p21 siRNA; B, Western blot respectively showing inhibition of p21 protein by p21 siRNA. C, % growth of the cells transfected with p27 siRNA; D, Western blot respectively showing inhibition of p27 protein by p27 siRNA. E, p27 deficiency and inhibition of heparin on PASMC proliferation. $p27^{+/+}$ and $p27^{-/-}$ mouse PASMC were incubated for 4 days with heparin. Cells grown in 10% FBS and 0.1% FBS were used for positive and negative controls. The results are representative of 3 separate experiments (total n=15), and error bars represent standard error. *$P<0.05$ as compared with 10% FBS. HP indicates heparin.

Loss of p27 Gene Negated the Inhibitory Effect of Heparin on PASMC Proliferation

To determine the role of p27 deficiency on PASMC proliferation, PASMCs transfected with p27 siRNA and PASMCs deficient in p27 were used. Gene silencing by introduction of p27 siRNA in PASMCs resulted in 70% inhibition of p27 protein expression and in the abrogation of heparin-dependent growth arrest of PASMCs, such that the growth of PASMCs was unabated ($P<0.05$ versus 10% FBS; Figure 2C and 2D). Similar to the results with p27 siRNA transfection, we did not observe an inhibitory effect of heparin on the growth of $p27^{-/-}$ PASMCs ($P<0.05$ versus 10% FBS; Figure 2E). These data demonstrate that the p27 gene was necessary for heparin-induced inhibition of PASMC proliferation. The scrambled p21 and p27 siRNA were used as controls for these siRNA experiments, but no gene silencing was observed (data not shown).

Deficiency of p21 Gene Does Not Attenuate the Inhibitory Effect of Heparin on Hypoxia-Induced Pulmonary Hypertension and Vascular Remodeling In Vivo

To determine the importance in the intact animal of the in vitro observations, we performed studies using p21-null mice. Heparin significantly inhibited the development of hypoxia-induced pulmonary hypertension in both $p21^{+/+}$ and $p21^{-/-}$ mice, as shown by RVSP(Figure 3A) and RV/LV+S (Figure 3B). Pulmonary vascular remodeling as shown by the wall thickness of the terminal bronchiolar arterioles (% WT-TA) and intraacinous arterioles (% WT-IA) and by the % thick of the intracinous vessels was significantly less in both $p21^{+/+}$ and $p21^{-/-}$ mice treated with heparin (Figure 4A through 4D). The value of the hematocrit was significantly higher in the hypoxic versus the normoxic groups, but no difference was observed between $p21^{+/+}$ and $p21^{-/-}$ hypoxic mice (Figure 3C). These results demonstrated that deficiency of the p21 gene did not prevent the inhibitory effect of heparin on hypoxia-induced pulmonary hypertension in vivo.

Hypoxia-Induced Pulmonary Hypertension Remodeling Was Inhibited by Heparin in Both $p27^{+/+}$ and $p27^{+/-}$ But Not in $p27^{-/-}$ Mice

Heparin reduced ($P<0.05$) RVSP and RV/LV+S in hypoxic wild-type ($p27^{+/+}$) and Het ($p27^{+/-}$) mice compared with hypoxic controls but not in p27 KO ($p27^{-/-}$) mice (Figure 5A and 5B). Heparin likewise reduced pulmonary vascular remodeling ($P<0.05$) when measured as % WT in terminal bronchial arterioles and intraacinous vessels and as % thick of intraacinous vessels in wild-type ($p27^{+/+}$) and Het ($p27^{+/-}$) but not p27 KO mice (Figure 6A and 6D). There were no

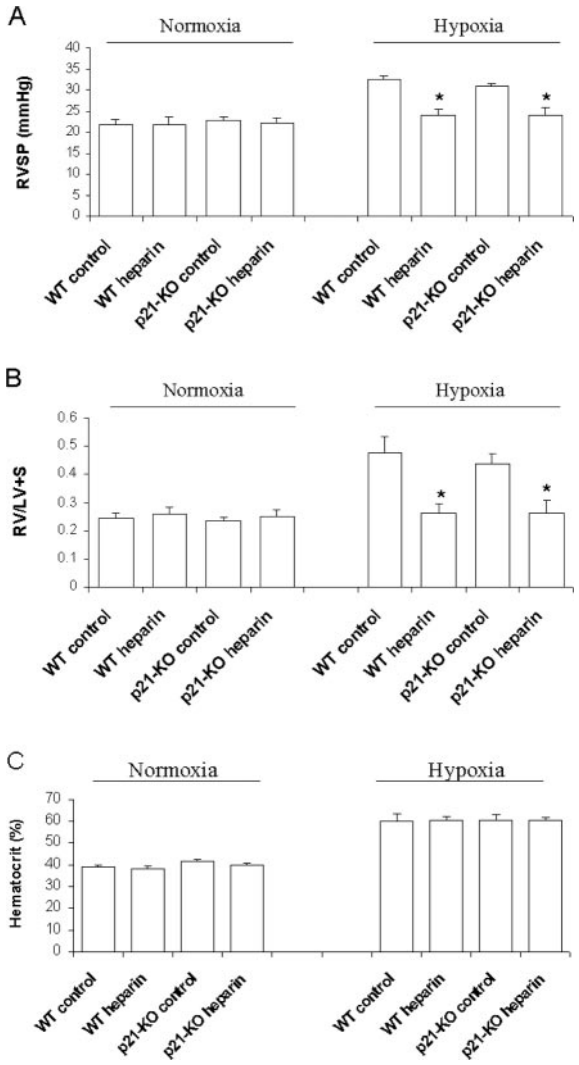


Figure 3. RVSP (A), RV/LV+S ratio (B), and hematocrit (C) in p21-deficient mice. WT control ($p21^{+/+}$ mice without heparin), WT heparin ($p21^{+/+}$ mice with heparin), p21-KO control ($p21^{-/-}$ mice without heparin), p21-KO heparin ($p21^{-/-}$ mice with heparin). n=6 for normoxia and n=8 for hypoxia. *$P<0.05$ as compared with littermate control mice.

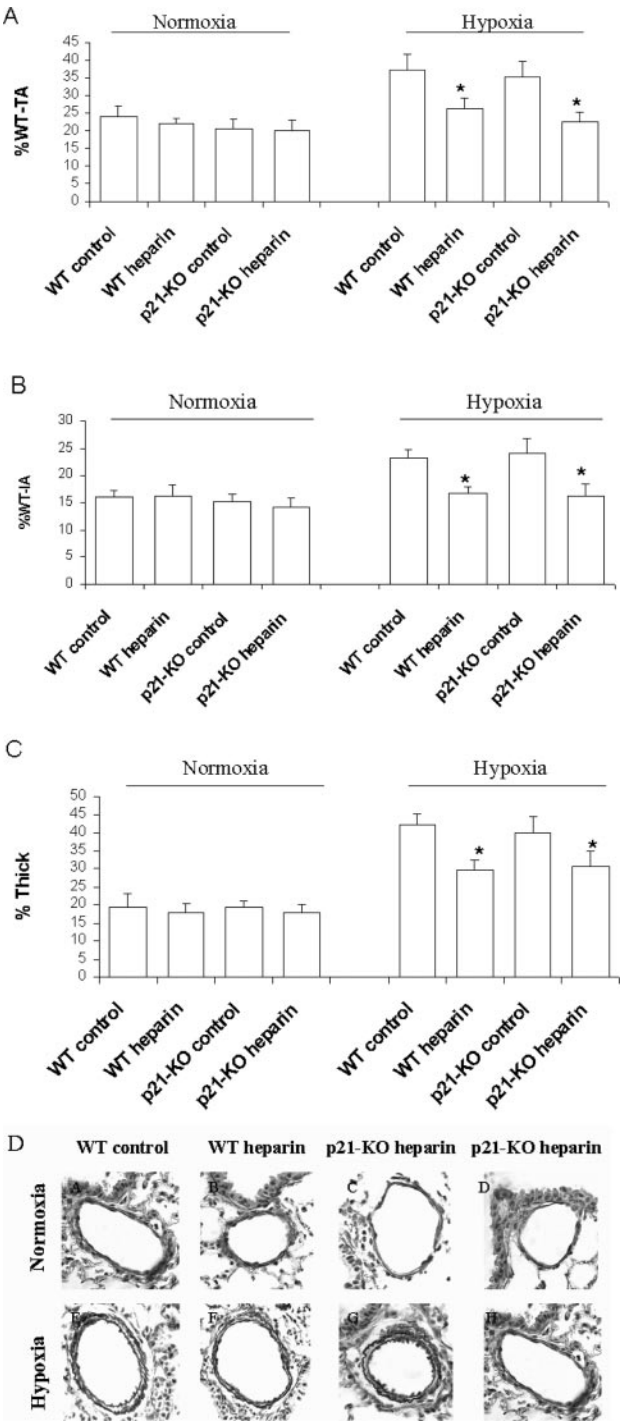


Figure 4. % WT-terminal bronchiolar arteriole (TA) (A), % WT-intracinar arteriole (IA) (B), % thick (C) in p21-deficient mice, and representative photomicrograph for terminal bronchial arterioles (D). WT control ($p21^{+/+}$ mice without heparin), WT heparin ($p21^{+/+}$ mice with heparin), p21-KO control ($p21^{-/-}$ mice without heparin), p21-KO heparin ($p21^{-/-}$ mice with heparin). n=6 for normoxia and n=8 for hypoxia. *$P<0.05$ as compared with littermate control mice.

significant differences in RVSP and vascular remodeling among any of the mice in normoxia with and without heparin treatment. Hypoxia caused a significant rise in hematocrit as compared with normoxia, but there was no significant difference among hypoxic groups (Figure 5C). These results indicated that p27 was required for the inhibitory effect of heparin on hypoxia-induced pulmonary hypertension in intact mice.

Hypoxia Decreased p27 Expression and Heparin Reversed This Decrease In Vivo

We found that hypoxia significantly decreased p27 mRNA and protein level in the lung and that heparin inhibited the decrease (Figure 7A). However, p21 expression was not affected by either hypoxia or heparin in the whole lung (Figure 7B).

Heparin Decreased Vessel Wall Ki67 Proliferative Index Induced by Hypoxia in WT and p27-Het, But Not in p27-KO Mice

To examine whether increased vascular wall cell proliferative activity correlated with the lack of effect of heparin observed

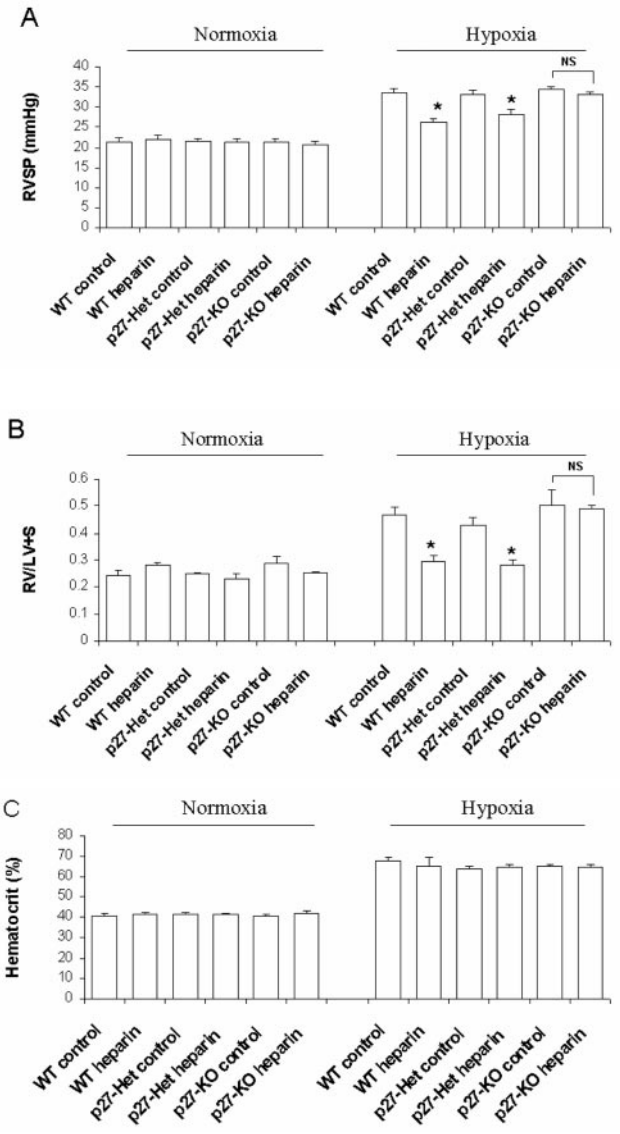


Figure 5. RVSP (A), RV/LV+S ratio (B), and hematocrit (C) in p27-deficient mice. WT control ($p21^{+/+}$ mice without heparin), WT heparin ($p27^{+/+}$ mice with heparin), p27-Het control ($p27^{+/-}$ mice without heparin), p27-Het heparin ($p27^{+/-}$ mice with heparin), p27-KO control ($p27^{-/-}$ mice without heparin), p27-KO heparin ($p27^{-/-}$ mice with heparin). n=5 for each groups. *$P<0.05$ as compared with littermate control mice. NS indicates not significant.

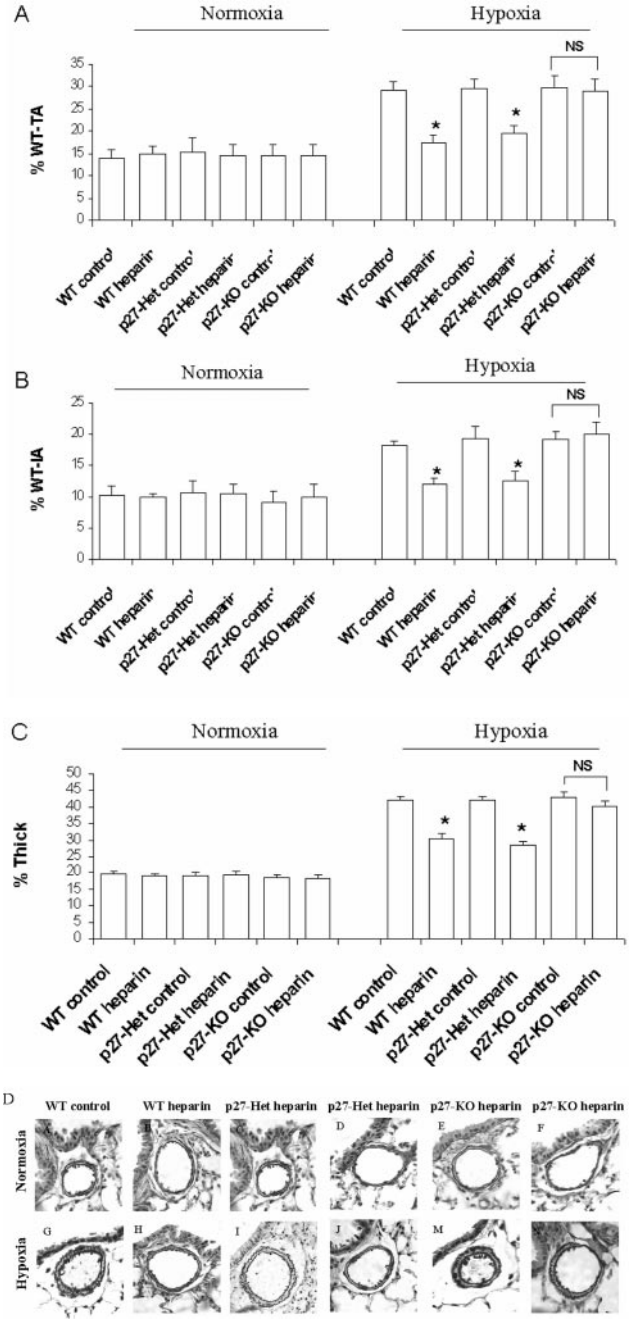
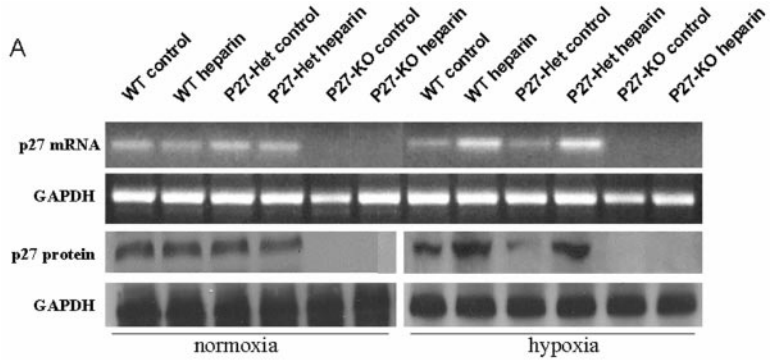


Figure 6. %WT-TA (A), %WT-IA (B), % thick (C) in p27-deficient mice, and representative photomicrograph (D) for terminal bronchial arterioles. WT control ($p27^{+/+}$ mice without heparin), WT heparin ($p27^{+/+}$ mice with heparin), p27-Het control ($p27^{+/-}$ mice without heparin), p27-Het heparin ($p27^{+/-}$ mice with heparin), p27-KO control ($p27^{-/-}$ mice without heparin), p27-KO heparin ($p27^{-/-}$ mice with heparin). n=5 for each groups. *$P<0.05$ as compared with littermate control mice. NS indicates not significant.

in hypoxic p27-null mice versus hypoxic wild-type and p27-Het mice, we compared the Ki67 proliferative index of vascular wall cells in the pulmonary vessels from normoxic and hypoxic animals. Fewer than 3.5% of the cells in the vessel wall of the terminal bronchiolar arteriole were Ki67-positive in normoxic mice with or without heparin, compared with ≈41% in hypoxic control mice. Specifically, there were 40.2±2.1 in WT, 41.0±1.6 in p27-Het and 42.1±1.2 in p27-KO mice, respectively. Heparin, however, decreased the % Ki67-positive cells to 26.5±1.7 in hypoxic WT and 27.3±1.3 in hypoxic p27-Het mice ($P<0.05$ versus littermate controls), but did not influence the Ki67 expression in hypoxic p27-KO mice, which was 41.3±2.0%.

Discussion

The present study demonstrates that p27 is critical to the prevention of pulmonary vascular smooth muscle hyperplasia by heparin in vitro (Figures 1 and 2) and hypoxia induced pulmonary hypertension in vivo (Figures 5 and 6). p21 was not important to this process (Figures 3 and 4).



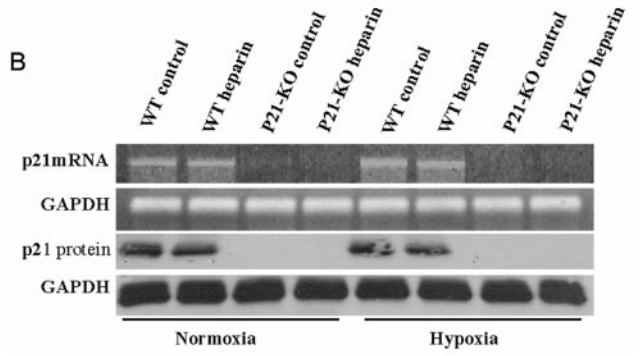


Figure 7. Expression of CDK inhibitors in mouse lungs. Total RNA and protein from the mouse lung tissue was isolated and then subjected to RT-PCR and Western blot analysis. GAPDH was used for equal loading control. WT control (wild-type mice without heparin), WT heparin (wild-type mice with heparin), p27-Het control ($p27^{+/-}$ mice without heparin), p27-Het heparin ($p27^{+/-}$ mice with heparin), p27- or p21-KO control ($p27^{-/-}$ or $p21^{-/-}$ mice without heparin), p27- or p21-KO heparin ($p27^{-/-}$ or $p21^{-/-}$ mice with heparin). The results are representative of 3 separate experiments. A, p21 expression in p21-null mice. B, p27 expression in p27-null mice. The results are representative of 3 separate experiments.

Heparin inhibition of SMC proliferation has been associated with several factors including the suppression of c-*fos* and c-*myc*, [26] inhibition of the EGF receptor,[27] nitric oxide synthesis,[28] protein kinase activity,[29] modulation of cytosolic calcium,[30] inhibition of the Na^+/H^+ exchanger,[19,25] as well as an increase in p21[11] and p27.[30] These data suggest that heparin inhibition of SMC proliferation probably involves several different pathways. Our study revealed that the CDK inhibitor p27 plays a critical role in mediating the antiproliferative property of heparin on hypoxia-induced pulmonary hypertension and remodeling, but p21 does not.

p27 is a member of CIP/KIP family of CDK inhibitors and inhibits cyclin E/CDK2 activity. In vitro overexpression of p27 decreases SMC proliferation,[15,31] and inhibition of p27 activity enhances baboon aortic SMC proliferation.[32] Fouty et al in their study of the role of the p27 gene in modulating PASMC proliferation used $p27^{-/-}$ PASMCs and found a 2-fold increase in [3H]thymidine incorporation and cell proliferation in $p27^{-/-}$ PASMCs compared with $p27^{+/+}$ PASMCs.[15] Tanner et al[17] also observed that overexpression of p27 caused a reduction of aortic SMC proliferation. In the present study, we found that inhibition of PASMC proliferation by heparin was accompanied by induction of both p27 mRNA and protein, and, furthermore, blockade of the p27 gene expression by p27 siRNA transfection of bovine PASMCs or by KO of the p27 gene in mouse PASMCs resulted in loss of the antiproliferative effect of heparin, thus demonstrating the importance of p27 in regulating PASMC proliferation.

p27 also plays a critical role in vivo in mediating cell growth, and disruption of p27 causes an alteration in cell proliferation.[24,33,34] Fero et al,[24] Kiyokawa et al,[33] and Nakayama et al[34] found that a lack of functional p27 resulted in increased animal size from continued cell proliferation. Sun et al[35] showed that the lack of p27 reduced rapamycin-mediated inhibition of SMC migration. Cool et al[36] reported that p27-negative cells occurred in pulmonary hypertension in the central core of plexiform lesions where the cells proliferate. p27-positive cells were present in the peripheral area adjacent to incipient blood vessel formation. These data show that p27 might be an essential element in regulation of pulmonary vascular cell proliferation. In our study, the $p27^{+/+}$, $p27^{+/-}$, and $p27^{-/-}$ mice developed similar pulmonary hypertension and vascular remodeling under hypoxia. However, $p27^{-/-}$ mice lost heparin-mediated inhibition of hypoxic pulmonary hypertension and vascular remodeling, suggesting p27 was an important cofactor for the inhibitory effect of heparin, though not sufficient by itself, to alter hypoxic pulmonary vascular remodeling. Interestingly, in our study the p27-Het mice ($p27^{+/-}$) developed pulmonary hypertension and vascular remodeling indistinguishable from p27 wild-type ($p27^{+/+}$) mice. This is in contradistinction to the results with tumorigenesis and atherosclerosis where possessing 1 allele of p27 is partially protective compared with wild-type ($p27^{+/+}$).[37,38]

Yu et al[39] and Hirst et al[40] observed increased Ki67 expression in cultured PASMCs and human bronchial SMC with growth factor stimulation. Roque and colleagues[41] reported that decreased Ki67 expression was correlated with an increase in p27 expression in a porcine coronary angioplasty model. We also investigated the cell proliferation marker Ki67 expressed in the medial wall of terminal bronchial arterioles to further determine whether heparin inhibition of PASMC proliferation in vivo is mediated by p27. Our results

revealed that PASMC proliferation in p27-KO mice was not affected by heparin although heparin inhibited cell proliferation in WT and p27-Het animals. This finding has provided additional support to the notion that the protective effect of heparin against hypoxic pulmonary vascular remodeling is mediated, at least in part, by p27-dependent growth arrest.

The p21 gene, another member of the CIP/KIP family of CDK inhibitors, also is an important modulator in the regulation of cell cycle progression. Overexpression of the p21 gene has been associated with a reduction in systemic artery SMC proliferation.[17] In addition, Khoury and Langleben[11] observed that heparin inhibition of pulmonary vascular pericyte proliferation caused by hypoxia was accompanied by induction of p21. Our data showed, however, that although heparin inhibition of PASMC proliferation was associated with upregulation of p21 in vitro, this increase in p21 was not necessary for the inhibition of cell growth because blockade of p21 gene expression by the use of p21 siRNA did not affect the inhibitory effect of heparin on PASMC proliferation. Heparin also inhibited the proliferation of both $p21^{+/+}$ and $p21^{-/-}$ hematocrit cells. With the use of p21-deficient mice, we further demonstrated that p21 was not critical for the inhibitory effect of heparin on hypoxia-induced pulmonary hypertension in mice.

We observed a different effect of heparin on induction of p21 mRNA and protein in vitro and in vivo. p21 was induced in the heparin treated PASMCs, but was not affected in the hypoxic heparin-treated mice. It may be that the p21 signal is not involved in regulation of hypoxia-induced pulmonary hypertension in vivo. This finding also indicates that not all in vivo findings can be predicated by in vitro studies.

The fundamental cellular mechanism of action of heparin on SMC growth and the precise structural determinants of the heparin mechanism required for its antiproliferative action remain unknown. Heparin does bind growth factors, and this may contribute to its mode of action by depriving cells of these growth stimuli.[42] Heparin, however, also appears to bind to specific receptors on the SMC surface, and the antiproliferative effect is enhanced >10-fold when quiescent SMCs are incubated with heparin for 48 hours before growth stimulation.[42] Internalization of receptor bound heparin appears to occur during this time, at least in part suggesting an intracellular site of action.[42,43] We have also shown that heparin can inhibit PASMC proliferation induced by serotonin, a growth factor that is not bound to heparin.[44] Though heparin binding to growth factors may be involved in the inhibition of PASMC proliferation, this is not the only mechanism.

In conclusion, our study reveals that the CDK inhibitor p27 plays a critical role in the inhibitory effect of heparin on hypoxia-induced pulmonary hypertension and remodeling. p21, another CDK inhibitor, was not necessary for the inhibition of hypoxia-induced pulmonary hypertension by heparin.

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