Heparin Inhibits Pulmonary Artery Smooth Muscle Cell Proliferation through Guanine Nucleotide Exchange Factor-H1/RhoA/Rho Kinase/p27

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Ras homolog gene family member A (RhoA) through Rho kinase (ROCK), one of its downstream effectors, regulates a wide range of cell physiological functions, including vascular smooth muscle cell (SMC) proliferation, by degrading cyclin-dependent kinase inhibitor, p27. Our previous studies found that heparin inhibition of pulmonary artery SMC (PASMC) proliferation and pulmonary hypertension was dependent on p27 up-regulation. To investigate whether ROCK, a regulator of p27, is involved in regulation of heparin inhibition of PASMC proliferation, we analyzed ROCK expression in the lungs from mice and from human PASMCs exposed to hypoxia, and investigated the effect of ROCK expression *in vitro* by RhoA cDNA transfection. We also investigated the effect of guanine nucleotide exchange factor (GEF)-H1, an upstream regulator of RhoA, on heparin inhibition of PASMC proliferation by GEF-H1 cDNA transfection. We found that: (1) hypoxia increased ROCK expression in mice and PASMCs; (2) overexpression of RhoA diminished the inhibitory effect of heparin on PASMC proliferation and down-regulated p27 expression; and (3) overexpression of GEF-H1 negated heparin inhibition of PASMC proliferation, which was accompanied by increased GTP-RhoA and decreased p27. This study demonstrates that the RhoA/ROCK pathway plays an important role in heparin inhibition on PASMC proliferation, and reveals that heparin inhibits PASMC proliferation through GEF-H1/RhoA/ROCK/p27 signaling pathway, by down-regulating GEF-H1, RhoA, and ROCK, and then up-regulating p27.

Keywords: Rho kinase; guanine nucleotide exchange factor-H1; pulmonary artery smooth muscle cell; proliferation; heparin

Ras homolog gene family member A (RhoA), a member of Rho GTPases, is found in all eukaryotic cells, and its downstream factor, Rho kinase (ROCK), play a wide range of fundamental cellular functions, such as contraction, migration, adhesion, proliferation, differentiation, and gene expression (1–3). Activated RhoA binds to ROCK to stimulate a downstream signaling pathway through the substrates of ROCK (3). It has been reported that proliferation of different cells, including vascular smooth muscle cells (SMCs), has been inhibited by a ROCK inhibitor (4).

Recent studies have shown that RhoA/ROCK signaling plays an important role in mediating pulmonary vasoconstriction, hypertension, and vascular remodeling (5–9), and pharmacologic inhibition of ROCK has been shown to block hypoxia-induced pulmonary hypertension and vascular remodeling (7–10).

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CLINICAL RELEVANCE

This study demonstrates that heparin inhibits pulmonary artery smooth muscle cell proliferation through the guanine nucleotide exchange factor-H1/RhoA/RhoA kinase/p27 signaling pathway. These results provide a new mechanism by which heparin inhibits pulmonary hypertension and vascular remodeling.

p27^{Kip1} (p27) is a primary negative regulator of cyclin-dependent kinase (CDK) in SMCs and plays an important role in inhibition of CDK activity (12). p27 inhibits phosphorylation of cyclin-CDK complexes, which results in inhibition of the activity of this complex and cell growth arrest in G1 phase (13, 14). Reports from some investigators have shown that activated ROCK can bind to and then degrade p27 protein, thereby regulating p27 activity (4, 15–18), leading to the acceleration of cell cycle progression (19).

Our previous studies have shown that antiproliferative heparins significantly inhibit pulmonary vascular remodeling induced by hypoxia in rodents (20–23) and pulmonary artery SMC (PASMC) proliferation in culture (24, 25). Other investigators also have reported that heparin inhibits PASMC proliferation *in vitro* and *in vivo* (26, 27). However, the mechanisms responsible for the antiproliferative effects of heparin are unknown in detail, although there is evidence that heparin inhibits cell proliferation via inhibiting activation of protein kinase C (PKC), extracellular signal-regulated kinases (ERK) 1/2, activator protein 1 (AP-1), *c-fos*, and *c-jun* (28). Recently, we have found that p27 plays a critical role in heparin inhibition of PASMC proliferation *in vitro* and pulmonary hypertension *in vivo* (14, 29). Fasciano and colleagues (30) have also reported that heparin inhibition of cell growth is dependent on p27 up-regulation *in vitro*. In addition, we recently found that decreased ROCK expression was accompanied by an increase in p27 expression in sodium/hydrogen exchanger isoform 1-deficient mice (31).

Guanine nucleotide exchange factors (GEFs) promote Rho activity through catalyzing the exchange of GDP for GTP to generate the activated form of Rho (32, 33). GEF-H1, which is associated with cytoskeletal structure, microtubules, and actin cytoskeleton, has recently been characterized as a RhoA-specific GEF (29, 46) involved in the regulation of RhoA activity (34–39).

Based on the role of RhoA/ROCK in hypoxia-induced pulmonary hypertension and the ability of ROCK to regulate p27 activity, we hypothesized that overexpression of RhoA/ROCK signaling will negate the effect of heparin on inhibition of PASMC proliferation and on up-regulation of p27. Therefore, in this study, we first examined the effect of heparin on RhoA/ROCK expression in mice with pulmonary hypertension and remodeling, and then conducted RhoA gene transfection to investigate the role of RhoA/ROCK in heparin inhibition of PASMC proliferation and up-regulation of p27 gene expression. We also investigated

the effect of GEF-H1, an upstream regulator of RhoA/ROCK, on heparin inhibition of PASM C proliferation.

MATERIALS AND METHODS

Detailed description of materials and methods is provided in the online supplement.

Animals

Mice were exposed to 10% hypoxia for 14 days and then removed for measurement of pulmonary hemodynamics and for collection of lung tissue for biological analysis.

Cells and Hypoxia Exposure

Bovine and human PASM Cs were cultured in normoxia or under 2% hypoxia.

Gene Transfection and Cell Growth Assay and Cell Cycle Analysis

PASM Cs were transfected with RhoA cDNA plasmid and GEF-H1 cDNA plasmid, and then cell proliferation assay and cell cycle analysis were performed after treatment of the cells with heparin.

RT-PCR and Western Blot

RNA and protein were extracted from cultured PASM Cs for RT-PCR and Western blot analysis of mRNA and protein expression.

GTP-RhoA Pulldown Assay

A GTP-bound RhoA pulldown assay was performed to determine RhoA activity by measuring GTP-RhoA.

Subcellular Fractionation and RhoA Assay

Subcellular protein fractions of the PASM Cs were extracted for measurements of RhoA and guanine nucleotide dissociation inhibitor (GDI) in different locations in the cytosol and membrane.

Statistical Analysis

Statistical analyses were performed using the computer program, Statview (SAS Institute Inc., Cary, NC) with ANOVA. If ANOVA indicated significance, multiple comparisons were made among groups using Fisher's protected least significant difference test. All values were

expressed as the mean ($\pm$ SE). Significance was set at a *P* value less than 0.05.

RESULTS

Hypoxic Exposure Increased ROCK Expression and Heparin Inhibited the Increase in Mice

A 2-week exposure to hypoxia significantly increased ROCK1 and -2 expression in mouse lungs as compared with normoxic animals. Heparin significantly reduced the increased ROCK expression in the hypoxic mice (Figure 1A). However, RhoA expression was affected by neither hypoxia nor heparin.

Hypoxic Exposure Increased ROCK Expression in Human PASM Cs

ROCK1 and -2 are two isoforms of ROCK encoded by two different genes, but are highly homologous. To determine if hypoxia impacts ROCK expression in PASM Cs, we exposed human PASM Cs to 2% hypoxia and then analyzed ROCK expression over the course of time. We found that hypoxia significantly increased protein expression of ROCK1 and -2 in a time course, but that the RNA expression was not affected (Figure 1B).

Overexpression of RhoA Diminished the Inhibitory Effect of Heparin on PASM C Proliferation

We subsequently performed a RhoA gene transfection to determine the role of RhoA/ROCK in regulating heparin inhibition of PASM C proliferation. After transfection of the bovine PASM Cs with RhoA constitutively active cDNA (RhoA-CA), an upstream factor of ROCK, and treatment with heparin, we found that proliferation of the PASM Cs was significantly inhibited by heparin in a dose-dependent manner in the PASM Cs transfected with empty plasmid (pcDNA3.1) or RhoA domain negative plasmid and in normal control cells. However, the proliferation of the PASM Cs transfected with RhoA-CA was not affected by heparin treatment (Figure 2A). These findings demonstrate that overexpression of the RhoA gene resulted in loss of heparin inhibition of PASM C proliferation.

Figure 1. Effect of heparin on Rho kinase (ROCK) expression in hypoxic mice, and effect of hypoxia on ROCK expression in human pulmonary artery smooth muscle cells (PASM Cs). (A) ROCK expression in hypoxic mice: total protein from mouse lungs was isolated and then subjected to Western blot analysis. Representative images of Western blot (*left*) and quantitative results (*right*), showing relative ROCK expression, setting normoxic control as 1. **P* < 0.05 as compared with other groups. (B). ROCK expression in human PASM Cs: representative images of RT-PCR and Western blot (*left*) and quantitative results (*right*). **P* < 0.05 as compared with previous time-point group. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for equal loading control. The results are representative of three separate experiments.

Heparin Affected the Level of GTP-RhoA in PSMCs

To investigate whether the decreased ROCK expression during heparin inhibition of PSMC proliferation influences RhoA activity, a pulldown assay to measure GTP-RhoA was performed for determination of RhoA activity. We found that GTP-RhoA was significantly decreased in the cells treated with heparin as compared with the control cells (Figure 2B). Because GTP-RhoA, an active form of RhoA, exists in cell membrane and GDP-RhoA, an inactive form of RhoA, is in cytosol (cytoplasmic matrix), we subsequently performed a subcellular fractionation of the cells to obtain RhoA in different components of the cell to determine the impact of heparin on the localization of RhoA in membrane versus cytosol. We observed a decrease in RhoA in the cell membrane and an increase in cytosol in the heparin-treated cells (Figure 2C).

Overexpression of RhoA/ROCK Inhibited Heparin Induction of p27

To investigate if p27 is involved in RhoA/ROCK pathway for heparin inhibition of PSMC proliferation, we analyzed p27 expression from the cells transfected with RhoA-CA. We found a significant increase in p27 expression in control PSMCs treated with heparin, which was consistent with the results that we observed previously (29). However, the RhoA-

CA transfection significantly inhibited p27 expression, and heparin treatment no longer reversed the down-regulation (Figure 2D).

Heparin Inhibited GEF-H1 Protein Expression

To address whether heparin affected RhoA activity through GEF-H1, an upstream regulator of RhoA, we measured GEF-H1 mRNA and protein expression from the lung of hypoxic mice. We found a significant decrease in the expression of GEF-H1 protein in the mice treated with heparin (Figure 3A). However, the expression of GEF-H1 mRNA was not significantly changed.

Overexpression of GEF-H1 Decreased Heparin Inhibition of PSMC Proliferation

Because we observed that GEF-H1 expression was inhibited by heparin, we hypothesized that heparin inhibited RhoA/ROCK through GEF-H1. To confirm this hypothesis, we performed GEF-H1 gene transfection using a wild-type GEF-H1 cDNA plasmid. We found that the PSMC proliferation was not significantly affected by heparin in the cells transfected with GEF-H1 cDNA, although heparin significantly inhibited cell growth in the control cells and in the cells transfected with empty plasmid in a dose-dependent manner (Figure 3B).

Figure 2. Effect of over expressing RhoA on heparin inhibition of PSMC proliferation and on RhoA activity and location, as well as p27 expression. (A) Overexpression of RhoA and heparin inhibition of PSMC proliferation. Cells grown in 10% FBS and 0.1% FBS were used for positive and negative controls. The cells in heparin treatment groups were grown in 10% FBS plus different doses of heparin (HP). The results are representative of three separate experiments (total $n = 15$). * $P < 0.05$ as compared with 10% FBS. (B) GTP-RhoA pulldown assay showing relative GTP-RhoA and total RhoA expression. Control was cells growing in 10% FBS (thereafter). (C) RhoA localization and (D) p27 expression. Representative images of Western blot (left) and quantitative results (right), setting control as 1. The results are representative of three separate experiments. * $P < 0.05$ as compared with control cells.

Figure 3. Effect of overexpressing guanine nucleotide exchange factor (GEF)-H1 on heparin inhibition of PASM C proliferation. (A) GEF-H1 mRNA and protein expression. Total RNA and protein from the lungs of hypoxic mice treated with or without heparin were isolated for RT-PCR and Western blot analysis. Representative images of RT-PCR and Western blot (left) and quantitative results (right), setting control as 1. * $P < 0.05$ as compared with control cells. The results are representative of three separate experiments. (B) GEF-H1 overexpression and PASM C proliferation. * $P < 0.05$ as compared with 10% FBS. The results are representative of three separate experiments (total $n = 15$). GEF-H1, GEF-H1 cDNA.

Overexpression of RhoA and GEF-H1 Blocked Heparin Inhibition of Human PASM C Proliferation and Cell Cycle Progression under Hypoxia

To demonstrate further the effect of RhoA and GEF-H1 on heparin inhibition of human cell proliferation under hypoxia, we used human PASM Cs and hypoxic condition. After transfection with RhoA cDNA and GEF-H1 cDNA, we exposed the cells to 2% hypoxia for 24 hours and found that overexpression of RhoA and GEF-H1 blocked the inhibitory effect of heparin on human PASM C proliferation (Figure 4A). This result was consistent with the data obtained from bovine PASM Cs under normoxia (Figure 2A). In the meantime, we found a significant decrease in S and G2/M phase and an increase in G0/G1 phase in the human PASM Cs treated with heparin. However, overexpression of both genes completely avoided the changes (Figure 4B).

RhoA and GEF-H1 Small Interfering RNA Silencing Inhibited Human PASM C Proliferation under Hypoxia

We subsequently transfected RhoA small interfering RNA (siRNA) and GEF-H1 siRNA into human PASM Cs separately, and found that both siRNAs significantly inhibited human PASM C proliferation under hypoxia (Figure 4C).

Overexpression of GEF-H1 and RhoA Impacted RhoA Activity and p27 Expression in Human Pasmcs under Hypoxia

After analyzing expression of GEF-H1, GTP-RhoA, and p27 in the human PASM Cs transfected with RhoA and GEF-H1 cDNAs, we found that overexpression of GEF-H1 significantly increased GEF-H1 and GTP-RhoA and decreased p27 (Figure 5A), and overexpression of RhoA significantly increased GTP-RhoA and decreased p27, but did not impact GEF-H1 (Figure 5B). In addition, we did not observe significant change in phosphorylation of p27 by heparin (Figure 5C).

Figure 4. Effect of overexpressing RhoA and GEF-H1 on human PASM C proliferation and cell cycle progression under hypoxia. (A) RhoA and GEF-H1 cDNA transfection and human PASM C proliferation, (B) cell cycle analysis, and (C) RhoA and GEF-H1 small interfering RNA (siRNA) silencing and PASM C proliferation. * $P < 0.05$ as compared with control. The results are representative of three separate experiments (total $n = 15$).

DISCUSSION

RhoA/ROCK activation has been related to hypoxia-induced pulmonary hypertension in animals (6, 7, 9–11, 40–42). Inhibition of ROCK activity by ROCK inhibitors has been shown to attenuate hypoxia-induced pulmonary hypertension (43). In the present study, we found an increase in expression of ROCK in the lungs from mice with hypoxia-induced pulmonary hypertension, and a hypoxic induction ROCK expression in human PASM Cs. Our results provide further supportive information on RhoA/ROCK in the hypoxia-induced development of pulmonary hypertension.

Investigations from different laboratories have found that administration of ROCK activity inhibitors significantly decreased the development of hypoxic pulmonary hypertension in animals (5, 7, 10, 11, 44). We recently found a decrease in ROCK activity in mice with deficiency of sodium/hydrogen exchanger isoform-1 gene, and the mice had significantly less hypoxia-induced pulmonary hypertension and vascular remodeling (31). Another report showed that simvastatin attenuation of pulmonary hypertension was accompanied by a greater inhibition of RhoA activity (45). Interestingly, in this study, we found that heparin, which has been shown to inhibit pulmonary hypertension and vascular remodeling, also significantly inhibited

Figure 5. Effect of overexpressing RhoA and GEF-H1 on GEF-H1, GTP-RhoA, and p27 in human PASMCs under hypoxia. Proteins were isolated from human PASMCs under hypoxia treated with GEF-H1 cDNA, RhoA cDNA, and heparin, respectively, for Western blot analysis (total protein for GEF-H1, p27, and GAPDH; GTP pull-down protein for GTP-RhoA). (A) Effect of GEF-H1 on GTP-RhoA and p27 expression. (B) Effect of RhoA on GEF-H1 and p27 expression. (C) Heparin and p27 phosphorylation. Representative images of Western blot (left) and quantitative results (right), setting control as 1. * $P < 0.05$ as compared with control group. The results are representative of three separate experiments.

ROCK activity in mice with hypoxia-induced pulmonary hypertension.

After we found that heparin inhibited hypoxia-induced ROCK expression, we questioned if RhoA/ROCK was necessary for heparin inhibition of PASC proliferation. We overexpressed RhoA in bovine PASCs and then treated the cells with heparin. We found that overexpression of RhoA resulted in the loss of heparin inhibition of proliferation in the PASCs in normoxia. These data demonstrate that the inhibition of RhoA/ROCK activity was necessary for heparin inhibition of hypoxic pulmonary hypertension and vascular remodeling.

Subsequently, we found that heparin decreased the level of GTP-RhoA, the active form of RhoA, in PASCs treated with heparin (see Figure 2B). We also found a decrease in RhoA expression in the cell membrane and an increase in RhoA in the cytosol in the cells treated with heparin (see Figure 2C). ROCK is tightly related to RhoA activity, in which activated RhoA binds to ROCK (ROCK1 and -2), and results in the activation of ROCK. The active RhoA, GTP-bound RhoA (GTP-RhoA), located on the cell membrane, will bind to and phosphorylate its downstream effector, ROCK. Therefore, the amount of GTP-RhoA in cells will determine the activity of ROCK. In this study, we found that heparin not only reversed the activity of hypoxia-induced ROCK (see Figure 1A), but also affected the GTP-transition of RhoA and the localization of RhoA in PASCs (see Figures 2B and 2C). These results indicate that heparin mediated RhoA activity through affecting the transition of GDP-RhoA to GTP-RhoA, a necessary step to stimulate ROCK.

We, in our previous studies, have found an essential role of p27, a cyclin inhibitor, in heparin inhibition of hypoxia-induced pulmonary hypertension and vascular remodeling (14, 29). Fasciano and colleagues (30) also reported that heparin inhibition of cell growth was dependent on p27 up-regulation. Previous reports from other investigators have shown that ROCK is an upstream regulator of p27 (4, 7, 15, 16, 18, 19). To ask if heparin up-regulation of p27 was dependent on RhoA/ROCK, we measured p27 expression from the PASCs transfected with or without RhoA gene. We found that heparin did not affect p27 expression in the cells transfected with RhoA gene, although heparin increased p27 expression in the control cells. We also found no significant change in p27 phosphorylation in the cells treated with heparin. These results demonstrate that heparin up-regulation of p27 was via the inhibition of RhoA/ROCK.

RhoA activity is controlled by GEFs and inactivated by GTPase-activating protein. GEFs catalyze exchange of GDP for GTP to activate the switch and GTPase-activating proteins stimulate the intrinsic GTPase activity to inactivate the switch (1). GEF-H1 is a RhoA-specific GEF (34, 35). In this study, we performed a GEF-H1 gene transfection to determine if GEF-H1 was involved in heparin inhibition of PASC proliferation. We found that transfection of the GEF-H1 gene also resulted in the loss of heparin inhibition of PASC proliferation. Because GEF is an important regulator of RhoA activity (34–39), these results reveal that heparin inhibition of PASC proliferation was also dependent on GEF-H1. This finding demonstrated that heparin regulated RhoA/ROCK through GEF-H1.

Figure 6. Effect of heparin on the GEF-H1/RhoA/ROCK/p27 signaling pathway in inhibition of PASM C proliferation. Under normal physiological condition, the GEF-H1/RhoA/ROCK/p27 signaling pathway regulates a variety of cellular functions, including cell proliferation. Growth stimulus, including hypoxia, can stimulate GEF-H1 activity, which catalyzes the change of GDP for GTP to generate GTP-RhoA. GTP-RhoA consequently activates its downstream effector, ROCK, and then ROCK degrades p27, which eventually results in PASM C proliferation and development of pulmonary hypertension. Heparin inhibits PASM C proliferation and hypoxic pulmonary hypertension through inhibiting GEF-H1, which results in a decrease in GTP-RhoA and ROCK. The decrease in ROCK attenuates degradation of p27, which then results in an increase in p27. *Dashed arrows*, growth stimulation; *solid arrows*, heparin treatment.

To demonstrate further the role of GEF-H1 and RhoA/ROCK in heparin inhibition of PASM C proliferation in human cells, we used human PASM Cs and performed studies to investigate cell proliferation and cell cycle progression under hypoxia using the same procedure for bovine cells. We found that the overexpression of HEF-H1 and RhoA also significantly blocked heparin inhibition of human PASM C proliferation under hypoxia. In the meantime, we found that the overexpression of RhoA and GEF-H1 negated an increase in G0/G1 phase and a decrease in S and G2/M phase induced by heparin. We also found that RhoA activity was increased by overexpressed RhoA and GEF-H1, but that GEF-H1 was not impacted by overexpressed RhoA. p27, a downstream effector of RhoA/ROCK, was significantly decreased by both overexpressed GEF-H1 and RhoA. We thus demonstrated the same results in human PASM Cs.

In regard to heparin regulation of GEF-H1, ERK, a member of the mitogen-activated protein kinases, might play an important role. Investigators have recently shown that GEF-H1 was regulated by ERK (46, 47). Fujishiro and colleagues (46) observed an enhanced GEF-H1 activity by ERK1/2 in cancer cells, which resulted in activation of RhoA to up-regulate cell proliferation and motility. Kakiashvili and colleagues (47) found that TNF- α induced kidney injury via the ERK/GEF-H1/RhoA/ROCK/phospho-myosin light chain (MLC) pathway, in which GEF-H1 was activated by ERK1/2 in kidney epithelial cells. Their studies indicated a regulatory effect of ERK on GEF-H1. Very interestingly, we previously reported a significant decrease in ERK1/2 in hypoxic mouse lungs induced by heparin, and

found that ERK1/2 mediated p27 expression in PASM Cs (14). Therefore, heparin may regulate GEF-H1 through ERK1/2.

In summary, the present study demonstrates that RhoA/ROCK and GEF-H1 play a critical role in heparin inhibition of PASM C proliferation that results in pulmonary hypertension and vessel remodeling. These findings suggest a novel mechanism, in which heparin inhibits pulmonary hypertension and vascular remodeling via the GEF-H1/RhoA/ROCK/p27 signaling pathway by down-regulating GEF-H1, RhoA, and ROCK, and then up-regulating p27 (Figure 6). The widely used model of hypoxia-induced pulmonary hypertension in rodents mainly causes PASM C proliferation and hyperplasia, which result in increased thickness of pulmonary artery wall. Although different mechanisms are involved in development of monocrotaline- and shunt-induced pulmonary hypertension, PASM C proliferation is also observed in these animal models. Therefore, the results from this study may also be comparable in other animal models of pulmonary hypertension involving PASM C proliferation. However, we cannot declare that this mechanism is also suitable for all other animal models of pulmonary hypertension at this time. Right ventricular hypertrophy and failure are the most severe consequence of pulmonary hypertension. Unfortunately, we did not specifically investigate the changes in ROCK in right ventricle in this study. Therefore, these results cannot be directly applied to right heart biology during pulmonary hypertension.

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