Role of nitric oxide in heparin-induced attenuation of hypoxic pulmonary vascular remodeling

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Heparin inhibits the development of pulmonary hypertension and vascular remodeling associated with prolonged hypoxia; however, the mechanism is not completely understood (8, 13, 15, 30). Continuous intravenous heparin (300 U·kg⁻¹·day⁻¹) infusion for 10 days of hypoxic exposure has been shown to attenuate increases in pulmonary arterial pressure (PAP), right ventricular hypertrophy, and pulmonary vascular remodeling in mice (8). This attenuation does not appear to be related to an anticoagulant effect of heparin because warfarin, also an anticoagulant, does not attenuate hypoxic pulmonary hypertension (11). The effect of heparin is specific to the pulmonary circulation because doses of heparin that decrease hypoxic pulmonary hypertension do not affect systemic hypertension (19). The effectiveness of different preparations of heparin to inhibit the development of hypoxic pulmonary hypertension in vivo appears to be related to its antiproliferative potency in vitro (30).

Heparin may attenuate the development of hypoxia-induced pulmonary vascular remodeling by inhibiting smooth muscle cell (SMC) growth. In vitro studies have demonstrated that heparin inhibits rat SMC proliferation (24, 30). Heparin has also been shown to have properties that specifically affect the vascular endothelial, which may act on the vascular smooth muscle.

Heparin potentiates acetylcholine-stimulated cGMP and nitric oxide (NO) formation as determined by nitrite/nitrate levels in rat cultured aortic endothelial cells (ECs) (23). However, Upchurch et al. (31) reported that high-dose heparin increases in vitro platelet aggregation in media conditioned by bovine aortic endothelial cells by decreasing endothelial NO production.

Increases in NO secondary to heparin may play an important mechanistic role in regulation of the pulmonary vasculature. Hypoxic pulmonary hypertension is attenuated by exogenous sources of NO. Inhaled NO (18, 27), L-arginine (23), NO donors (28), and inhibitors of cGMP degradation (3) decrease pulmonary vascular remodeling and attenuate increases in PAP secondary to hypoxia. Additionally, endogenous NO is a negative regulator of vascular smooth muscle proliferation (25).

This study investigated the hypothesis that heparin-induced attenuation of hypoxic pulmonary vascular remodeling is NO mediated. In rats, the effect of a continuous infusion of heparin (1,200 U·kg⁻¹·day⁻¹) on pulmonary vascular remodeling was evaluated after 3 days and 3 wk of exposure to 10% O₂. The role of NO in heparin-induced attenuation of pulmonary vascular
remodeling was determined by infusion of the NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME). Endothelial NOS (eNOS) protein, NOS activity, and cGMP were measured to determine the effects of heparin on the NO-cGMP pathway in the lungs. In vitro, the effects of heparin on cGMP levels and SMC proliferation were studied in an EC-SMC coculture model under normoxic and anoxic conditions.

**METHODS**

*In vivo experiments.* This study was approved by the University of Virginia Animal Care and Use Committee. Male Sprague-Dawley rats (n = 8 per group), weighing 270–350 g, were evaluated for 3-day or 3-wk exposure periods. Environmental exposure was either normobaric normoxia (21% O2) or normobaric hypoxia (10% O2). Hypoxic groups were treated with or without heparin (1,200 U·kg⁻¹·day⁻¹; sodium salt from bovine lung, Sigma Chemical, St. Louis, MO) and/or L-NAME (20 mg·kg⁻¹·day⁻¹; Sigma Chemical) dissolved in 0.9% saline (Baxter, Deerfield, IL). Solutions were administered via an Alzet osmotic pump (model 2ML4, Alza, Palo Alto, CA) implanted subcutaneously in the dorsal mid-scapular region. Rats were allowed 24 h of recovery from the surgical procedure before administration of drugs and being placed in the environmental chambers.

Maintenance of the hypoxic environment was performed as previously described (27). Rats were placed in a Plexiglas chamber, and O2 levels were regulated with a Proox model 350 unit (Reming Bioinstrument, Redfield, New York) and maintained at 10% O2 by infusion of N2.

After 3 days or 3 wk of environmental exposure, the rats were removed from the chambers and immediately anesthetized with 0.4 g urethane and 30 mg α-chloroethane. An open-chest measurement of PAP was made by inserting a 22-gauge catheter into the right ventricle and advancing it into the pulmonary artery. The systolic and diastolic pressures were measured by an epidural catheter into the right ventricle and advancing it into the pulmonary artery. The systolic and diastolic pressures were maintained at 10% O2 by infusion of N2.

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Additional rat lungs (n = 8 per group) were snap frozen to measure NOS activity and cGMP levels. NOS activity was determined by measuring the formation of L-[3H]citrulline from L-[3H]arginine as previously described (5). Enzymatic reactions were terminated by adding 2 ml ice-cold stop buffer containing 20 mM sodium acetate (pH 5.5), 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA. The L-[3H]citrulline produced was separated from L-[3H]arginine by Dowex AG 50W-X8 (Na⁺ form, Bio-Rad Laboratories) column. cGMP was extracted by homogenizing tissue in 0.1 N ice-cold hydrochloride. After centrifugation, the supernatant was analyzed for cGMP by radioimmunoassay (Amersham).

*Cell culture.* DMEM + F12 (DMEM-F12), MEM, fetal bovine serum (FBS), penicillin, streptomycin, trypsin-EDTA, and PBS were obtained from Gibco (Grand Island, NY). Vascular SMCs (rat, Sprague-Dawley, established) were maintained in DMEM-F12 + 10% FBS, penicillin-streptomycin, and l-glutamine (0.4 g/500 ml). Pulmonary artery ECs (bovine) were maintained in MEM, 10% FBS, 1% penicillin-streptomycin, and 0.4% thymidine. cGMP was measured by radioimmunoassay (Amersham).

*BrdU incorporation assay.* SMCs were plated in 24-well plates at a density of 3 × 10⁵ cells/cm² and grown in DMEM+F12 with 10% FBS and 1% penicillin-streptomycin. The medium was aspirated 24 h after SMC plating. Endothelial cells grown to confluency on microcarrier beads were diluted in 1:1 MEM-DMEM-F12 medium with 5-bromo-2′-deoxyuridine (BrdU; 10⁻⁵ M) and plated on the confluent SMC layer. Heparin (1, 10, and 100 U/ml) and L-NAME (10⁻⁵ M) were added to treatment wells. Plates were placed in modular incubator chambers and purged with 5% CO₂-balance N₂ (anoxia) or 5% CO₂-balance air (normoxia) for 20 min. After 3 h of incubation at 37°C, medium was aspirated from the wells and the cells were washed twice in PBS (pH 7.4). Cells were fixed in 3% paraformaldehyde in PBS for 90 min. Cells were washed twice in PBS and incubated in 0.1 N HCl for 1 h at 37°C. The solution was aspirated, and 1.0 N HCl was added for 30 min. Cells were washed twice in PBS, and 0.1 ml of primary antibody solution was added to each well for a period of 2 h. The primary antibody solution contained 1:100 rabbit anti-human Von Willebrand factor (vWF; Dako, Glostrup, Denmark), 1:100 monoclonal mouse anti-BrdU (Dako) and 1:20 normal goat serum (NGS, Sigma Chemical) in 0.4% Triton (Sigma Chemical), and 3% bovine serum albumin (Sigma Chemical) in deionized water. ECs were identified by positive reaction with the vWF primary antibody. BrdU-positive nuclei indicated cells in S phase during the 3-h exposure period. NGS was used to block
unspecific binding of antibodies. After a wash in PBS, cells were then incubated for 1 h in 0.1 ml/well secondary antibody solution. Secondary antibody solution contained 1:200 AMCA-Fab (Jackson ImmunoResearch Laboratories, West Grove, PA), 1:200 Cy3 anti-rabbit Ig (Jackson ImmunoResearch Laboratories) and 1:20 NGS in 0.4% Triton (Sigma Chemical), and 3% bovine serum albumin (Sigma Chemical) in deionized water. Secondary antibody solution was aspirated, and a solution of 1:200 IA4-FITC (Sigma Chemical) and 1:5,000 Sytox green nucleic acid stain (Molecular Probes, Eugene, OR) was added for 1 h. IA4-FITC stained α-actin positive cytoplast and identified SMC, whereas Sytox stained all dead nuclei.

To quantify SMC cell DNA synthesis, the percentage of BrdU-positive, α-actin-positive, vWFP-negative nuclei per total nuclei was determined for each well. At least 500 but not more than 600 total nuclei were counted per well by using a FITC filter and a ×20 objective on a Zeiss Axioscope (Thornwood, NY). The image was observed on a Sony monitor connected to the Axioscope via a video intensifier (Dage MTI, Michigan City, IN) in series with a charge-coupled device camera (Dage MTI). After total nuclei in a given field of view were counted, BrdU-positive cells were counted by using an AMCA filter. Fields of view were selected for counting if the total number of SMC nuclei was at least 10 but no greater than 100 to avoid variability in proliferation associated with local seeding density. After videotaping of each field of view, the images were digitized and analyzed by using the Optimus (v6.1) software package.

Data analysis. Body weight, RV/(LV+S), and PAP were determined from the mean of all rats within a group. Percent muscularization (%M) was determined for 50 vessels examined in each lung section. %T was assessed for each muscular vessel, and the mean %T of all muscular vessels from an individual lung section was used in determining the group mean. Densitometric results from Western blots were normalized to normoxic control values. SMC proliferation as assayed by BrdU-incorporation was determined by the percent BrdU + SMC per total SMC nuclei (%BrdU + SMC). Data were analyzed by one-way ANOVA with SigmaStat software (Jandel Scientific). Individual comparisons between group means were made with a t-test with Bonferroni’s correction factor for multiple tests. Significance was assumed at P < 0.05. Data are expressed as means ± SE.

RESULTS

In vivo results. Pilot studies determined that there were no significant differences in PAP or RV/(LV+S) between rats with saline-filled osmotic pumps and rats without pumps in either normoxic or hypoxic rats after 3 wk.

After 3 days of hypoxia, RV/(LV+S) was unaltered compared with normoxia (0.31 ± 0.01 vs. 0.27 ± 0.02). Hypoxic rats administered heparin (0.34 ± 0.02), l-NAME (0.36 ± 0.02), or the combination (0.35 ± 0.03) were also not significantly different from normoxia at 3 days. Three weeks of hypoxia significantly increased RV/(LV+S) compared with normoxic controls (Fig. 1). Although heparin significantly attenuated this increase in hypoxic rats, RV/(LV+S) remained greater than normoxic controls. l-NAME alone did not significantly alter RV/(LV+S) in hypoxic rats at 3 wk; however, l-NAME prevented the attenuation of RV/(LV+S) caused by heparin.

Three days of hypoxia in the presence (11 ± 2 mmHg) or absence (10 ± 2 mmHg) of heparin had no effect on PAP compared with normoxia (10 ± 2 mmHg). Although l-NAME alone (14 ± 1 mmHg) had no effect on hypoxic rats, l-NAME and heparin significantly increased PAP (20 ± 3 mmHg) compared with 3-day hypoxic controls. After 3 wk of hypoxia, PAP was significantly increased compared with normoxic controls (Fig. 1). Heparin significantly attenuated the increase in PAP compared with hypoxic controls. Although l-NAME alone had no effect in hypoxic rats, l-NAME prevented the attenuation of PAP secondary to heparin.

Vascular remodeling was assessed by the proportion of pulmonary vessels classified as muscular (%M) and the thickness of the medial layer of muscular vessels as a percentage of their short-axis diameter (%T). After 3 days of exposure, hypoxia with or without heparin or l-NAME had no significant effect on %M or %T. After 3 wk of hypoxia, both %M and %T were significantly increased (Fig. 2). Heparin significantly attenuated this increase. l-NAME attenuated the effects of heparin in hypoxic rats, whereas l-NAME alone did not significantly affect %M or %T.

NOS activity was significantly increased by hypoxia after 3 days and 3 wk compared with normoxic controls. After 3 days and 3 wk, heparin significantly increased NOS activity compared with hypoxia alone. There was no difference between the 3-day and 3-wk heparin plus hypoxia groups. The cGMP levels were also significantly increased by hypoxia after 3 days and...
3 wk (3 wk > 3 days) compared with normoxic controls. Heparin increased cGMP levels at 3 days and 3 wk compared with hypoxia alone, with the 3-wk heparin plus hypoxic values greater than the 3-day heparin plus hypoxic group. L-NAME abolished NOS activity and cGMP levels (Fig. 3).

The eNOS protein level was significantly increased by hypoxia after 3 days and 3 wk compared with normoxic controls (Fig. 3). Heparin increased eNOS at 3 days compared with hypoxia alone. However, after 3 wk, eNOS was not different from normoxic controls. The eNOS level in the hypoxic group with L-NAME and heparin was not different from hypoxia alone at either 3 days or 3 wk but was greater than normoxic controls. L-NAME had no effect on eNOS levels in hypoxic rats.

In vitro results. The cGMP levels were evaluated in an EC-SMC coculture after incubation periods of 10 min and 24 h under normoxic and anoxic conditions. After a 10-min normoxic or anoxic incubation period, endothelial-dependent and -independent positive controls, bradykinin and sodium nitroprusside, increased cGMP levels five- and twofold, respectively. Heparin, at concentrations of 10 and 100 U/ml, significantly increased cGMP levels after 10 min of normoxia and hypoxia compared with normoxic controls (Fig. 4). There was no significant difference between the two concentrations. The cGMP levels after 10 min were not different between anoxic and normoxic groups.

After 24 h of normoxia, the cGMP levels were significantly increased compared with 10 min of normoxia for all groups. The cGMP levels were significantly less for all anoxic groups compared with the normoxic groups. After 24 h, heparin, at concentrations of 10 and 100 U/ml, significantly increased cGMP levels under both normoxic and anoxic conditions. L-NAME decreased the cGMP levels to near zero.

The SMC proliferation was assessed by %BrdU+ after 3 h. Heparin significantly decreased proliferation during the normoxic incubation period compared with normoxic controls (Fig. 5). L-NAME alone had no significant effect in normoxic conditions, and L-NAME did not reverse the effects of heparin. Anoxia significantly decreased SMC proliferation compared with normoxic controls. Heparin and/or L-NAME did not significantly affect proliferation under anoxic conditions.

**DISCUSSION**

This in vivo and in vitro study investigated whether the mechanisms by which heparin attenuates hypoxic pulmonary vascular remodeling are mediated by NO. Heparin attenuated the increase in PAP, RV/(LV+S), %M, and %T associated with 3 wk of hypoxia. Importantly, NOS inhibition with L-NAME prevented this attenuation secondary to heparin. A role for NO is further suggested by the observation that heparin increased lung NOS activity and cGMP levels at 3 days and 3 wk and increased eNOS protein levels at 3 days. In cocultures, heparin increased cGMP levels; however, heparin decreased cell proliferation by a mechanism that appears to be NO independent.

Heparin significantly attenuated the increase in %M and %T associated with 3 wk of hypoxia but not with 3 days of hypoxia. The results at 3 wk are consistent with other studies, whereas the lack of an effect at 3 days is most likely related to the time required to observe significant hypoxia-induced changes in the pulmonary vasculature. Hales et al. (8) showed that heparin (300 U·kg⁻¹·day⁻¹) significantly attenuated pulmonary artery hypertension, RV/(LV+S) and remodeling of distal small pulmonary arteries in mice exposed to hypoxia for 3 wk. In contrast, Hu et al. (13) found that heparin (720 U·kg⁻¹·day⁻¹) did not attenuate hypoxia-induced right ventricular hypertrophy after only 10 days of hypoxia.

L-NAME alone did not significantly affect pulmonary vascular remodeling in hypoxic rats. Although L-NAME has previously been shown to acutely increase PAP in normal and hypoxic rats (27), chronic L-NAME does not appear to alter pulmonary vascular remodeling after 3 wk of hypoxia. This is consistent with Hampl et al. (9), who reported that chronic hypoxia, but not L-NAME, induced pulmonary vascular remodeling. L-NAME alone did not alter PAP at 3 days or 3...
wk; however, after 3 days the PAP was increased in the heparin plus L-NAME hypoxic rats. L-NAME is known to cause greater vasoconstriction in the presence of elevated NO levels (27), a finding that occurs with hypoxia plus heparin at 3 days. However, this vasoconstriction is not apparent at 3 wk as measured by PAP. The most important finding in this study is that L-NAME prevented the effect of heparin on PAP, RV/(LV+S), %T, and %M in 3-wk hypoxic rats. When administered alone, however, L-NAME did not have a significant effect on these parameters. These results suggest that the ability of heparin to attenuate pulmonary vascular remodeling in hypoxic rats is dependent on L-NAME.

Fig. 3. Nitric oxide synthase (NOS) activity (A; citrulline formed in nM·mg protein⁻¹·min⁻¹), cGMP levels (B; fmol/mg protein), and endothelial NOS (eNOS protein; C) in lung tissue. Solid bars, 3 days of hypoxia (H); open bars, 3 wk of hypoxia. Values are means ± SE. *Increased by 3 days and 3 wk of hypoxia, P < 0.05. #Heparin increased eNOS protein (3 days only), NOS activity, and cGMP levels at 3 days or 3 wk compared with hypoxic control, P < 0.05. †3 wk greater than 3 days. L-NAME abolished NOS activity and cGMP but had no effect on eNOS protein levels.

Fig. 4. cGMP levels after 10 min (A) and 24 h (B) in endothelial cell-smooth muscle cell coculture after exposure to heparin. Solid bars, normoxic coculture; open bars, hypoxic coculture. Values are means ± SE. *Heparin significantly increased cGMP levels in normoxic and hypoxic coculture at concentrations of 10 and 100 U/ml, P < 0.05.

Fig. 5. Smooth muscle cell proliferation after exposure to heparin and L-NAME. Solid bars, normoxia; open bars, anoxia. %BrdU, percent 5-bromo-2′-deoxyuridine (BrdU) + smooth muscle cell per total smooth muscle cell nuclei. Values are means ± SE. #Anoxia decreases smooth muscle cell (BrdU) incorporation compared with normoxia, P < 0.05. *BrdU incorporation is decreased by heparin alone and with heparin + L-NAME in normoxia compared with control, P < 0.05. Heparin and/or L-NAME had no effect in anoxia compared with anoxia control.
on NO. Previous studies in guinea pigs by Hassoun et al. (11) and Thompson et al. (30) indicate that the ability of heparin to attenuate pulmonary vascular remodeling is not related to heparin’s anticoagulant effects. Heparin may decrease pulmonary vascular remodeling by increasing NO, which causes both vasodilation and antiproliferative effects. Studies on human veins suggest heparin stimulates NO and subsequently induces vasodilation. The decrease in vascular tone caused by heparin has been shown to be attenuated by the NO and cGMP inhibitors l-NMMA (29) and methylene blue (12). Heparin may also modulate SMC proliferation by an NO-mediated mechanism. The anti-mitogenic effects of NO on SMCs have been well documented in vivo and in vitro (6, 7). Mechanisms of action are likely to include both direct effects, which may be both cGMP-dependent and cGMP-independent, and indirect effects on factors such as SMC migration and death (12).

Our study indicates that the NO-cGMP pathway is stimulated by heparin in vivo. Heparin significantly increased lung NOS activity and cGMP levels at 3 days and 3 wk and increased eNOS protein levels after 3 days. This increase in NOS activity, eNOS protein, and cGMP was greater than for hypoxia alone, a factor that has previously been shown to stimulate the NO-cGMP pathway (32). Heparin-induced increases in NOS activity is consistent with results by Kouretas et al. (16), who investigated the effects of heparin on endothelial cells and isolated vascular rings and demonstrated that heparin increased eNOS activity. Their study also suggested that the mechanism involves a pertussis toxin-sensitive inhibitory G protein. In a study of the effect of heparin on gastric ulcer healing, heparin dose-dependently increased eNOS content in the blood vessels of the mucosa and submucosa. Although eNOS was increased, the expression of eNOS mRNA was not, suggesting modulation occurs at the level of translation rather than transcription (17). However, these findings are contradicted by Bachetti et al. (1), who reported a decrease in aortic eNOS protein expression and impairment of NO-dependent vascular reactivity after heparin infusion in rats. It is unlikely that inducible NOS plays a significant role because our laboratory’s previous studies have indicated that it is minimally present compared with eNOS in normoxic or hypoxic rats (5).

Whereas heparin increased lung NOS activity and cGMP levels at both 3 days and 3 wk, eNOS protein levels were increased at 3 days but decreased at 3 wk. The decreased eNOS at 3 wk may be related to negative feedback of increased NO on eNOS protein (26). The increase in NOS activity at 3 wk may explain the increase in cGMP levels despite the decrease in eNOS protein. Stimulation of the NO-cGMP pathway by heparin was completely abolished by 1-NAME as measured by NOS activity and cGMP levels, which indicates the effectiveness of 1-NAME in blocking the NO-cGMP pathway in these experiments. This observation, in conjunction with data demonstrating that 1-NAME attenuated the effects of heparin in vivo, strongly suggests an important role of NO in heparin-induced attenuation of hypoxic pulmonary vascular remodeling.

The effect of heparin on NO in an EC-SMC coculture was indirectly assayed by measuring cGMP levels. The decreased cGMP levels associated with anoxia is consistent with reports showing an inhibitory effect of hypoxia on eNOS mRNA levels and cGMP production in human umbilical vein ECs after 24–48 h (22) and in bovine pulmonary ECs (21). Importantly, heparin (10 and 100 U/ml) significantly increased cGMP levels after 10 min and 24 h of incubation in both normoxic and anoxic cells. Although increased cGMP production at 24 h may be a consequence of induction of NOS protein expression (19), the increase found after 10 min is likely a result of increased eNOS activity (16). Although the effect of heparin on cGMP production under anoxic conditions has not been previously investigated, our results demonstrate that heparin increases cGMP levels in the in vitro coculture model to a similar extent in both anoxic and normoxic conditions.

The effects of heparin and NO inhibition on SMC proliferation in the EC-SMC coculture model were determined indirectly by measuring BrdU incorporation. Heparin significantly decreased SMC proliferation under normoxic conditions. This is consistent with the in vivo results and suggests that heparin may attenuate vascular remodeling by inhibiting smooth muscle cell proliferation. However, in contrast to the in vivo results, 1-NAME did not significantly alter the effects of heparin on proliferation in coculture despite blocking the increase in cGMP. This result suggests that inhibition of smooth muscle cell proliferation by heparin may not be mediated by NO. Although these results are in apparent conflict with the in vivo results that suggest the mechanism of action of heparin is NO mediated, it is possible that the action of heparin is not solely mediated by NO. The studies by Tangphao et al. (29) and Hawari et al. (12) in human veins proposed that heparin-induced vasodilation is dependent on increased bioavailability of NO. This speculation is supported by a recent finding that heparin inhibits the generation of reactive O2 species that bind NO and reduce the bioavailability of NO (4). It is also likely that the presence of shear stress and pressure in vivo contribute to differences with in vitro results. In contrast to its antiproliferative effect in normoxic conditions, heparin had no effect on SMC proliferation in anoxia. However, this may reflect the dramatic decrease in cell proliferation secondary to anoxia that does not allow for unmasking of the effects of heparin in this model.

In conclusion, the in vivo portion of this study suggests that the attenuation of hypoxic pulmonary vascular remodeling by heparin is NO mediated. The effects of heparin on PAP and pulmonary vascular remodeling are attenuated by NOS inhibition with 1-NAME. Furthermore, lung NOS activity and cGMP levels are increased and eNOS protein levels are transiently increased by heparin. Similar effects were demonstrated in vitro because heparin increased cGMP.
levels under normoxic and anoxic conditions. However, the in vitro effects of heparin on SMC proliferation are not evident under anoxic conditions and do not appear to be NO mediated under normoxic conditions.

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