HIGH LEVELS OF HYALURONAN IN IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION

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Abstract

Hyaluronan (HA), a large glycosaminoglycan found in the extracellular matrix, has major roles in lung and vascular biology and disease. However, its role in idiopathic pulmonary arterial hypertension (IPAH) is unknown. We hypothesized that HA metabolism is abnormal in IPAH. We measured the plasma levels of HA in IPAH and healthy individuals. We also evaluated HA synthesis and the expression of HA synthases and hyaluronidases in pulmonary artery smooth muscle cells (PASMCs) from explanted lungs. Plasma HA levels were markedly elevated in IPAH compared to controls [HA (ng/ml, mean±SD): IPAH 325±80, control 28±9; p=0.02]. In vitro, unstimulated IPAH PASMCs produced high levels of HA compared to control cells [HA in supernatant (ug/ml, mean±SD): IPAH 12±2, controls 6±0.9; p=0.04]. HA levels were also higher in IPAH PASMC lysates. The increased HA was biologically relevant as shown by tissue staining and increased HA-specific binding of mononuclear cells to IPAH compared to control PASMCs [Number of bound cells x10⁴ (mean±SD): IPAH 9.5±3, control 3.0±1, p=0.01]. This binding was abrogated by the addition of hyaluronidase. HA synthase2 and hyaluronidase2 were predominant in control and IPAH PASMCs. Interestingly the expressions of HA synthase2 and hyaluronidase2 were about 2 fold lower in IPAH compared to controls [HA synthase2: IPAH 4.3±0.02, control 7.8±0.1; p=0.0004; hyaluronidase2: IPAH 4.2±0.06, control 7.6±0.07; p=0.008]. Thus, patients with IPAH have higher circulating levels of HA, and PASMCs derived from IPAH lungs produce more HA compared to controls. This is associated with increased tissue levels and increased binding of inflammatory cells suggesting a role for HA in remodeling and inflammation in IPAH.

Keywords: IPAH, hyaluronidase, lung, hyaluronan, remodeling
**Introduction**

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive disease that leads to deterioration in cardiopulmonary function and premature death from right ventricular failure (14, 17, 24, 26). While the pathogenesis of IPAH is not entirely known, the main processes believed to lead to progressive pulmonary arterial narrowing include vasoconstriction, *in situ* thrombosis, cellular proliferation, and ultimately vascular remodeling (14, 17, 24, 26). Although pulmonary artery smooth muscle cells (PASMCs) are considered a major component of the remodeling process in IPAH, the nature of the primary abnormality that triggers and perpetuates PASMC proliferation in IPAH is unclear. Extracellular matrix (ECM) has important roles in cell proliferation and migration. The integrity and balance of matrix components are essential for normal lung function and response to injury. Identifying matrix abnormalities in IPAH will help us better understand the disease pathobiology and may help efforts to resolve or reverse the disease process. Different lung diseases, like asthma, emphysema, and pulmonary fibrosis are associated with abnormal ECM turnover (45). There are several different components in the extracellular matrix of the lung, including proteoglycans, elastin and collagen. Proteoglycans consist of a core protein to which one or more glycosaminoglycan chains (GAGs) are attached. GAGs present in the lung include hyaluronan (HA), heparan sulfate, dermatan sulfate, and chondroitin sulfate (39). In addition to their importance for organ structure, matrix components are involved in mediating a variety of physiologic and pathologic processes. HA degradation products can induce the expression of a variety of genes, chemokines, cytokines, growth factors, signal transduction molecules and adhesion molecules (43). Furthermore, HA degradation products can stimulate angiogenesis (50).
HA is a large glycosaminoglycan composed of repeats of two alternating sugar units, β-D-N-acetylglucosamine and β-D-glucuronate and is part of pericellular and extracellular matrices. It is present in synovial fluids, the vitreous body, and several other tissues including the lungs (3). HA is synthesized by HA synthases (HAS), a group of three cell transmembrane proteins: HAS1, HAS2, and HAS3. These enzymes lengthen HA by repeatedly adding glucuronic acid and N-acetylglucosamine alternately to the nascent chain as it is extruded through the cell membrane into the extracellular space (25, 38). HAS expression is context specific according to cell type. All inhibitors of protein synthesis such as cycloheximide treatment, endoplasmic reticulum (ER) stress, and double-stranded RNA induce HA production in smooth muscle cells (20). The synthesis of HA is also increased in smooth muscle cells after viral infection or treatment with polyinosinic acid:polycytidylic acid (poly I:C), a synthetic double-stranded RNA that initiates responses similar to viral infection (9, 20).

While it is abundant in extracellular matrices, HA also contributes to proliferation of cells, and participates in a number of cell surface receptor interactions, including its primary receptor, CD44. HA's contribution to cell proliferation may be due to its interaction with CD44, which participates in cell adhesion interactions required by proliferating cells. The binding of CD44 to HA stimulates angiogenesis and aggregation, proliferation and migration of cells (6, 10, 13, 28). There is also evidence that HA degradation products may have inflammatory properties independent of interaction with CD44 (43).

Thus, HA has important roles in the lung response to injury and other pathobiologic processes implicated in IPAH, including angiogenesis, vascular remodeling, and cell proliferation and migration. However, the role of HA in IPAH has not been studied previously. Here we present the novel finding that IPAH patients have high levels of circulating HA. Our
data also show that one possible source of HA is PASMCs from IPAH lungs, which unlike PASMCs from controls, spontaneously produce high levels of HA without stimulation and make HA cables that have the functional ability to bind inflammatory cells. Our data suggest a potential role for HA in remodeling in and the interaction between inflammatory cells and smooth muscle cells in IPAH that contributes to the pathobiology of the disease.
Experimental Procedures

Study population- The study included 22 patients with IPAH identified based on the NIH registry diagnostic criteria for pulmonary hypertension subclass 1.1 according to the World Health Organization criteria (17, 40) and 9 healthy control volunteers. Demographic and clinical characteristics of the IPAH individuals are listed in Table 1. The healthy controls (mean age 32±2 years, 6 female) were individuals with no history of pulmonary or cardiac disease or symptoms. The study was approved by the Cleveland Clinic Institutional Review Board (IRB), and all participants signed an IRB-approved consent form prior to participation in the study.

Hyaluronan (HA)- Levels of HA in plasma or conditioned media were measured with a commercial competitive binding kit (R&D Systems, Minneapolis, MN, Cat no: DY3614). In these experimental conditions, the lowest detectable level of HA was 1.15 ng/ml. The assay was used according to manufacturer’s instructions.

Cell preparation and isolation- Human pulmonary artery smooth muscle cells (PASMC) were isolated from elastic pulmonary arteries (>500 micron diameter) dissected from lungs obtained at explantation during lung transplant. After removal of endothelial cells, PASMCs were dissociated by digestion with collagenase type II/DNAase I solution overnight at 37°C (52). Cells were cultured on uncoated plates in smooth muscle cell growth medium (SmGM-2, Cambrex) containing 5% glucose, 10% FBS and 5% Antibiotic-Antimycotic from GIBCO (Cat no: 15240). Cells were passaged at 60-90% confluence by dissociation from plates with 0.05% trypsin, 0.53 mM EDTA. Primary cultures of passage 5-8 were used in experiments. The smooth muscle phenotype of cultured cells was confirmed (> 97% purity) by immunohistochemistry and flow cytometric analysis with antibodies against smooth muscle alpha-actin and calponin.
Histological studies- lung tissues were taken from explanted lungs and fixed and embedded in paraffin, and 4 micron sections were prepared. The sections were stained with hematoxylin and eosin (H&E) for morphological examination.

Immunostaining- In cell culture, staining was done to detect HA by a HA binding protein probe (Calbiochem, cat no: 385911). PASMCs were seeded on culture slides (BD Falcon, Bedford, MA). Slides were rinsed once with Hanks’ balanced salt solution (BSS), fixed in -20°C methanol and air dried. The culture slides were preincubated with Hanks’ BSS with 2% fetal bovine serum (FBS) for 30 min at room temperature. After discarding the medium, culture slides were incubated in Hanks’ BSS with biotinylated HA binding protein at the recommended dilutions overnight at 4°C on a wet towel to prevent drying. After overnight incubation, the culture slides were washed 3 times with Hanks’ BSS without FBS and then incubated with fluorescein-tagged streptavidin (1:500) in Hanks’ BSS with 2% FBS for 60 min at room temperature. The culture slides were washed 3 times with Hanks’ BSS without FBS and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA). The edges of culture slides were then sealed with nail polish, and the slides kept at -20°C. Fluorescence images were collected using an HCX Plan Apo 40X, NA 1.25 oil immersion objective on a Leica DMR upright microscope (Leica-Microsystems, Wetzlar GmbH) equipped with a Retiga Exi cooled CCD Camera (Q Imaging, Burnaby BC Canada) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

In lung tissue, HA was also detected by the same HA binding protein probe used as described above. SMCs were stained with monoclonal anti-smooth muscle actin antibody (Sigma, cat no: A 2547) and endothelial cells were stained with polyclonal rabbit anti-human von Willebrand factor (vWBF) antibody (Dako, cat no: A0082). Confocal images were collected
using an HC PL APO 20X/0.7 N.A. or HCX PL APO 40X/1.25 N.A. objective lens on a Leica TCS-SP2-AOBS Confocal Microscope (Leica-Microsystems, Wetzlar GmbH). The excitation (Ex) / emission (Em) wavelengths were as follows: DAPI, Ex 351 nm, Em 400-480 nm, Alexa Fluor 488, Ex 488 nm, Em 500-550 nm, Alexa Fluor 568, Ex 561 nm, Em 570-630 nm, Alexa Fluor 633, Ex 633 nm, Em 640-800.

**Fluorophore-assisted carbohydrate electrophoresis (FACE) assays** - Proteinase K
digestion of cell layer fractions: PASMCs were grown in 6 well plates until they reached ~90% confluence. Some cultures were treated with polyI:C (20 ug/ml) for 18 h before the assay. The cells in 6 well plates were kept at -20°C until digestion with proteinase K (Invitrogen, Cat no: 25530-015, Carlsbad, CA). For proteinase K digestion, 100 mM ammonium acetate, pH 7 was added to each well. 125 ug/ml proteinase K was added and incubated at 60°C for 2 h with mixing every 30 min. Afterwards, another 125 ug/ml proteinase K was added followed by incubation for another 2 h. The digests were transferred to 1.5 ml microcentrifuge tubes and concentrated by vacuum centrifugation to ~250 ul. 1 ml of -20°C 100% alcohol was added to each sample followed by vortexing and overnight incubation at -20°C. Samples were then centrifuged at 14,000g for 20 min. After discarding the supernatant, pellets were washed with 1 ml -20°C, 75% alcohol. Samples were centrifuged again at 14,000g for 20 min, and the pellets were air dried at room temperature after discarding the supernatants. 0.1 M ammonium acetate, pH 7, was added to each sample followed by incubation at room temperature for 20 min. Samples were placed in a boiling water bath for 5 min to destroy remaining proteinase K.

**Chondroitinase and Hyaluronidase digestion**: 0.6 ul of 1% glacial acetic acid was added to each sample to optimize enzymatic action at pH 5-6. Hyaluronidase and chondroitinase ABC (Seikagaku, Cat no: 100741-1A and Cat no: 100330-1A respectively) were pooled together in a
1:1 ratio, and 3.2 ul of the enzyme mixture was added to each sample followed by incubation at 37°C for 3 h. The enzymes were heat inactivated as described for proteinase K. 160 ul -20°C 100% alcohol was added to each sample, and the samples were incubated overnight at -80°C. They were then centrifuged at 14,000g for 20 min. Supernatants that contained disaccharide digestion products from HA and chondroitin sulfate were dried by centrifugal evaporation in microtubes. A solution containing (12.5 mM 2-aminoacridone (AMAC) (Molecular Probes, Cat no: A-6289), (7.5% glacial acetic acid and 0.5 M cyanoborohydride (Aldrich, Cat No: 15.615-9) was added to each sample followed by incubation overnight at 37°C in the dark.

**FACE analysis:** At the end of the incubation, the samples were each mixed with 80% glycerol (Fisher Chemicals, New Jersey, Cat no: BP 229-1). Each sample was subjected to electrophoresis on mono composition gels with mono running buffer (300V at 4°C for 1 h). An ultra Lum transilluminator (365 nm) was used for gel imaging, and a Quantix cooled charge-coupled device camera (Roper Scientific/photometrics) was used to capture images by the Gel-Pro Analyzer program version 3.0 (Media Cybernetics). Image J (NIH, USA version 1.38x) was used to analyze the images.

**Assay for monocyte adhesion:** Monocyte binding to PASMCs was quantified as previously described (8, 9). PASMCs were grown to confluence in 24 well plates. Treatment with (polyI:C) as a positive control (20 ug/ml) was done 18 h before assay. Up to 70x10⁶ U937 cells per ml were labeled for 90 min at 37°C with 100 uCi $^{51}$Cr as sodium chromate (PerkinElmer Life Sciences) in 1 ml RPMI with 10% FBS. The radiolabeled U937 cells were washed 3 times with culture medium without serum. After washing the cells were resuspended to 1x10⁶/0.5 ml. 1x10⁶ labeled U937 cells were added to each well followed by incubation for 1 h at 4°C for the binding phase. All cultures were washed once with cold serum-free medium. To determine
whether the binding is mediated through HA, 200 µg/ml hyaluronidase (SigmaAldrich, Cat no: H4272) was added in certain wells. All wells were washed 2 more times with cold medium to remove non-adherent monocytes before lysis by 200 µl of 1 % Triton X-100. 100 µl of 1 % TritonX-100 was removed for quantification of radioactivity by 1470 Automatic Gamma Counter from Perkin Elmer. The number of U937 cells per well was calculated from the initial specific activity (8).

*cDNA synthesis and real time quantitative PCR (RTQ-RT-PCR)*- RTQ-RT-PCR was used to quantitatively measure Hyal1, Hyal2, HAS1, HAS2, HAS3 mRNA expression in normal and IPAH PASMCs. RNA was isolated from PASMCs obtained from explanted lungs. For cDNA synthesis, 1 µg of each RNA sample was digested with DNaseI (Roche Diagnostics, Manheim, Germany), and the respective mRNA probes synthesized using an oligo dT primer and MLV (Invitrogen Cat no: 18418-020). SYBR Green Technology (Applied Biosystems, Warrington, UK, Cat no: 4309155) was done for all RTQ-RT-PCR experiments. Reactions were done in an iCycler Thermal cycler (Bio-Rad) in a total volume of 20 µl using the annealing temperature in Table 2. Expressions of Hyals and HASs mRNA relative to the housekeeping gene GAPDH were calculated using the ΔΔCt method (31). The PCR conditions were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 65°C for 1 min for all primers. Hyal1 and Hyal2 primers were described previously (37).

*Western blot:* Expression of KDEL and hyaluronidase in PASMCs was evaluated by Western blot analysis. PASMCs from the patients with IPAH and control were cultured on P100 cell culture plates until they reached 70 % confluence. The cells were removed from the plates, pelleted by centrifugation, and lysed for 30 min on ice in a lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 % NP-40, and 10 % glycerol) containing 10 µg/ml pepstatin, 1 mM
PMSF, 1 mM DTT, 20 ug/ml aprotinin, 5 ug/ml leupeptin, 1 uM sodium orthovanadate. The cell lysates were incubated at 95\(^\circ\)C for 10 min with a 1% SDS-PAGE sample buffer with 10 % β-mercaptoethanol. SDS-polyacrylamide gel electrophoresis was done using 4-15 % gradient gels. The proteins were transferred to nitrocellulose as described previously (53), incubated with the primary anti-KDEL antibody or hyaluronidase antibody and developed using enhanced chemiluminescence (ECL) (Amersham). The antibodies used were the mouse anti-KDEL antibody (dilution 1:1000) purchased from Stressgen Biotechnologies Corp. (Victoria, Canada) and the rabbit anti-hyaluronidase-2 antibody (dilution 1:500) (a generous gift from Robert Stern, University of California, San Francisco).

**Hyaluronan Sizing** - PASMCs were lysed with 2% CHAPS (in 0.05 M Tris, pH 7.0) for 5 min. An equal volume of 8 M guanidine HCl (in 0.05M Tris, pH 7.0 and 1% CHAPS) was added. Cell lysates were incubated overnight at -20 after adding 4x cold 200 proof ethanol. Samples were centrifuged at 14,000 g for 10 min and washed with 75% ethanol. After air drying for 20 min they were resuspended in 100 mM ammonium acetate (pH 7.0) for 20 min at room temperature. To remove all traces of guanidine HCl the ethanol precipitation step was repeated. After the second ethanol precipitation, the pellet was digested with 1 mg/ml protease K in 0.01%SDS at 60\(^\circ\)C overnight, followed by speed-vac to 250 ul and adding 1 ml cold absolute ethanol and incubated at -20\(^\circ\)C overnight. After repeating ethanol precipitation, 100 ul of 100 mM ammonium acetate (pH 7.0) was added, and protease activity was destroyed by heating the samples at 100\(^\circ\)C for 5 min. One of each sample was used to quantify DNA with the PicoGreen dsDNA quantification reagent (Molecular probes, Cat no: P-11496). Samples were digested with 0.06 U/ul DNAse (Ambion, Cat no: 2224) and 0.04 ug/ul RNAse A (Roche, Cat no: 109169) overnight at 37\(^\circ\)C. After repeating the precipitation step, samples were resuspended in 20 ul of
100 mM ammonium acetate (pH 7.0). Samples were then divided in half and 1 ul hyaluronidase (Seikagu, Cat no: 100740-1) was added to one half of each sample and incubated overnight at 37°C for a negative control. The samples were dried by speed-vac and resuspended in 10 ul of 1x TAE buffer. Samples were subjected to electrophoresis on a 1% agarose gel alongside hyaluronan standards of different sizes, mega molecular weight HA (megaHA) (6100, 4570, 3050, and 1520 kDa) high molecular weight HA (HiHA) (1510, 1090, 966, 572, 495 kDa), lower molecular weight HA (LoHA) (495, 310, 214, 110 kDa). The gel was stained with 0.005% Stain-All overnight and then de-stained with water until clear before a picture was taken.

Hyaluronidase activity- Hyaluronidase activity was detected by hyaluronan zymography. Plasma from IPAH and control patients were diluted with 10 volumes of 0.15 M NaCl. The diluted samples were mixed with an equal volume of Laemmli’s sample buffer containing 4% SDS and no reducing reagent. The mixtures were applied to 8% SDS- polyacrylamide gels containing 0.17 mg/ml HA. After electrophoresis at 25 mA, the gels were rinsed with 2.5% Triton X-100 for 2 h at room temperature. The gels were incubated with fresh incubation buffer (0.1 M sodium formate, 0.15 M NaCl at pH 3.5) 16 hours at 37°C. Following incubation, the gels were rinsed with water and incubated with 0.1 ug/ml Actinase E in 20 mM Tris-HCl (pH 8.0) for 2 h at 37°C. The gels were rinsed again and incubated with 0.5% Alcian blue in 25% ethanol and 10% acetic acid for 30 min and destained with 25% ethanol and 10% acetic acid. Gels were photographed, and the agarose gel images were analyzed with ImageJ (National Institutes of Health, Bethesda, Maryland).

Statistical Analysis- All statistical analysis was done using Jump JMP (NC, USA) version: 5.0.1.2 for Windows. Continuous variables were compared with the independent two tailed t-test. P ≤ 0.05 was considered as significant.
Results

*HA levels in the plasma of the patients with IPAH and healthy control individuals:* HA concentrations in plasma were measured in 22 IPAH and 9 control individuals. As shown in Figure 1A, plasma levels of HA in IPAH patients were markedly higher compared to controls (HA (ng/ml, mean±SD): IPAH 325±80, controls 28±9; p=0.02).

*Hyaluronan in IPAH and control lung tissues:* In order to evaluate the presence and location of HA in IPAH lung tissue, we stained tissues from 3 explanted IPAH lungs and 3 control lungs. Figure 2 shows histopathology and immunostaining of similar size (100-200 um) arteries from IPAH and control lungs. In addition to H&E staining (Figure 2 A and B), the same tissue sections were also stained for cell nuclei (DAPI), smooth muscle cells (smooth muscle actin), endothelial cells (von Willibrand factor), and hyaluronan (HA binding protein) (Figure 2 G and H). While this method of HA staining is not quantitative, there appeared to be more intense HA staining (green) around the pulmonary arteries in IPAH (Figure 2 C and G) compared to controls (Figure 2 D and H). The negative controls stained with the secondary antibody only, showed slight background auto-fluorescence in the elastic lamina, which is expected (Figure 2 E and F). Figure 3 shows the same staining of a plexogenic lesion in an IPAH lung. While there is widespread staining for HA (green) surrounding the lesion, the area of most intense staining seems to correlate to areas of new collagen deposition on the H&E staining (closed arrows in Figure 3 A, B, and C).

*Unstimulated IPAH pulmonary artery smooth muscle cells produce high levels of HA compared to controls:* HA concentrations in the supernatants of 3 IPAH and 3 healthy control PASMC cultures were determined by the same method used for plasma. HA levels in the supernatants of PASMCs were significantly higher in IPAH cells compared to healthy controls:
[HA (ug/ml, mean±SD): IPAH 12±2, controls 6±0.9; p=0.04] (Figure 1B). Fluorescence light microscopic imaging was also used to qualitatively determine the synthesis of HA cables in IPAH and control PASMCs. Immunofluorescence pictures (Figure 4A and B) of fixed PASMCs from IPAH and control samples show that IPAH PASMCs produce more HA. Unstimulated IPAH PASMCs produced HA in the pericellular coat (arrow heads) and in extracellular cables (arrows). In order to quantify the difference in HA production between IPAH and control PASMCs, HA levels were measured by fluorophore-assisted carbohydrate electrophoreses (FACE). Without any treatment each cell layer was harvested, and the glycosaminoglycans were digested to disaccharides. After labeling the disaccharides fluorescently, they were subjected to electrophoresis. Figure 4C demonstrates that IPAH PASMCs have significantly higher HA levels compared with control PASMCs. [Image density on FACE gel (mean±SD): IPAH 10800±2000, controls 5200±880; p=0.001] (Figure 4D).

Inflammatory cells bind to HA produced by IPAH PASMCs: As shown in Figure 5A and B, U937 cells from a monocytic cell line (open arrows) bind to HA cables (closed arrows) produced consistently by IPAH PASMCs. There are few cables produced by PASMCs from control lungs and hence limited or no U937 cell binding. We confirmed this qualitative observation, by quantifying the binding of radiolabeled leukocytes in an adhesion assay (43) as shown in Figure 5C. U937 binding to IPAH PASMCs was more than 3 fold higher compared with control PASMCs [Number of bound cells x 10^4 (mean±SD): IPAH 9.5±3, control 3.0 ±1, p=0.01]. Mononuclear cell binding was increased by poly I:C treatment of IPAH and control to the same level. This binding is HA specific as it is abrogated by bovine testicular hyaluronidase treatment (Figure 5C).
Expression of HASs in PASMCs: Because the results of conventional PCR did not yield robust signals (data not shown), we used RTQ-RT-PCR to assess the expression HA synthase levels in PASMCs from three different control and three different IPAH lungs. The primer sequences used to detect HAS1, HAS2 and HAS3 and their accession numbers are shown in Table 2. HAS2 was the predominantly expressed enzyme in both IPAH and control PASMCs with levels that are ~10 times higher than either HAS1 or HAS3. Interestingly, HAS2 expression in IPAH PASMCs was only about half of the levels seen in control PASMCs [HAS2: IPAH 4.3±0.02, control 7.8± 0.1; p=0.0004] (Figure 6A). Thus, high HA contents in IPAH PASMCs are not explained by differences in HAS mRNA expression.

Expression of hyaluronidase isoforms in PASMCs: We examined hyaluronidase isoforms (Hyal1 and Hyal2) expression in PASMCs cultures derived from 3 control and 3 IPAH lungs by RTQ-RT-PCR. The primer sequences used to detect Hyal1 and Hyal2 isoforms and their accession numbers are shown in Table 2. Hyal2 expression was significantly higher than Hyal1 expression in both IPAH and control PASMCs (p<0.001). Interestingly, the expression level of Hyal2 in IPAH PASMCs was significantly lower compared to levels in control PASMCs [Hyal2: IPAH 4.2±0.06, control 7.6±0.07; p= 0.008] (Figure 6B). This suggests that decreased degradation of HA may contribute to the high levels of HA in IPAH.

Protein levels of hyaluronidase: We examined hyaluronidase protein levels in PASMC cultures derived from 3 control and 3 IPAH lungs by western blot analyses. The protein levels of hyaluronidase in IPAH PASMCs were similar to control PASMCs (Figure 7).

Hyaluronidase activity: In vivo plasma hyaluronidase activity was significantly lower in IPAH individuals compared to controls (Figure 8 A and B) [Image density on Hyaluronidase activity gel (mean±SD): controls 71955±4104, IPAH 53998±7125; p=0.04]. In vitro
Hyaluronidase activity was also lower in the supernatants from IPAH compared to control PASMCs [Image density on Hyaluronidase activity gel (mean±SD): controls 33889±2141, IPAH 26938±937; p=0.04] (Figure 8 C and D). These suggest that high level of HA in IPAH patients could be due to low activity of hyaluronidase.

**Hyaluronan sizing:** Since the size of HA can determine its physiological effects, we evaluated the size of HA produced by PASMCs. The size of HA was similar (range 3050 to 4570 kDa) in the cell lysates of IPAH and control PASMCs (Figure 9). In IPAH, there was also another less intense band in the range of 110 to 214 kDa. The HA levels were higher in IPAH despite equal loading of samples (DNA: IPAH PASMCs 0.261 ug/ml, control PASMCs 0.219 ug/ml), supporting the data obtained by FACE analysis (Figure 4C).

**Endoplasmic Reticulum (ER) stress:** The majority of ER resident proteins are retained in the ER through a retention motif composed of four amino acids at the end of the chaperone protein sequences. The most common retention sequence is KDEL (lys-asp-glu-leu). During the ER stress response these resident proteins are synthesized and secreted into the cytoplasm (53). To show whether continuous HA production from IPAH PASMCs is related to ER stress, we studied the expression of KDEL sequence containing proteins Grp 78 and Grp 96 in PASMCs from patients with IPAH and controls. Figure 10 is a western blot of protein expression from 3 IPAH and 3 control PASMC lysates. Expression of the KDEL sequence containing proteins Grp 78 and Grp 96 was not different between IPAH and control PASMCs. This suggests that high HA production in IPAH PASMCs is not related to ER stress which is known to stimulate the HA cable response (32).
Discussion

The pathogenesis of IPAH is not fully understood. The disease is typified by SMC proliferation and vascular remodeling. Although PASMCs are considered a major component of the remodeling process in IPAH, the processes by which PASMCs affect vascular proliferation and remodeling are not entirely clear. Our data suggest that HA metabolism by PASMCs in IPAH is abnormal resulting in high levels of HA in plasma and likely in their ECM. Enhanced interaction of inflammatory cells with an increased HA matrix may contribute to the pathobiology of IPAH.

The key findings in our study are that patients with IPAH have significantly higher levels of circulating HA when compared to controls. Furthermore, unstimulated PASMCs from IPAH patients produce HA cables that are capable of promoting mononuclear cell binding.

The levels of HA in the plasma of our controls were similar to normal plasma HA concentrations in adults reported in the literature (22, 30). In contrast, we found markedly increased HA concentrations (~10 fold) in plasma HA from patients with IPAH. These levels are similar to those reported in inflammatory conditions, including inflammatory bowel disease, asthma, and atherosclerosis (1, 9, 16, 29, 42, 47).

There are several possible explanations for the high levels of circulating HA in IPAH. In the lung, analysis of bronchoalveolar lavage (BAL) fluid from patients with sarcoidosis, idiopathic pulmonary fibrosis and asthma revealed high HA levels related to the intensity of the alveolitis and with clinical severity (42). It was postulated that the presence of HA in BAL fluid in interstitial lung diseases may reflect fibroblast activation and/or proliferation (4). Since fibroblast activation is not a known major feature of IPAH, this is an unlikely mechanism in our patients. High HA levels have also been reported in liver cirrhosis. Therefore, decreased
clearance of HA by the liver or the kidney, the major routes of clearance of circulating HA (12, 29) may be another reason for elevated circulating HA levels. While patients with severe IPAH may develop passive liver congestion and renal insufficiency, there was no evidence of liver cirrhosis or renal failure in any of our patients. All of our patients had normal liver function tests and creatinine levels. Our study population was limited to IPAH, and no cases of portopulmonary hypertension were included.

In order to better understand the source of these markedly elevated circulating HA levels in IPAH, we evaluated HA production by PASMCs and pulmonary artery endothelial cells (PAECs) from IPAH and control lungs. In our experiments PAECs from IPAH and control lungs in culture did not produce significant levels of HA (data not shown). Interestingly, however, PASMCs from IPAH lungs spontaneously produced higher levels of HA and synthesized HA cables without stimulation. This was distinctly different compared to PAECs from IPAH and control lungs and to PASMCs from controls.

Since HA levels likely reflect a balance between production and degradation, we evaluated the expression levels of the various HAS enzymes that are responsible for HA synthesis and the Hyals that are responsible for its degradation. The fact that the predominant HAS (HAS2) and Hyal (Hyal2) were the same in PASMCs from both controls and IPAH likely reflects the known tissue specificities of these enzymes. Interestingly, in PASMCs from IPAH lungs the expression of both HAS2 as well as Hyal2 were only approximately half of the levels seen in PASMCs from controls while Hyal2 protein levels by western analysis were similar in PASMC from IPAH and control lungs. Hyaluronidase activity in the plasma, however, was significantly lower in IPAH patients. This suggests that the high HA levels in IPAH are more likely due to decreased degradation than to increased production.
These findings confirm abnormalities in HA metabolism in PASMCs in IPAH, which brings up the question regarding the cause of these unusual abnormalities. Possibilities include inherent abnormalities in the PASMCs themselves in IPAH or abnormalities in the lung environment. While both possibilities are plausible, our data could support both explanations.

In support of inherently abnormal PASMCs in IPAH, PASMCs from IPAH retain their ability to accumulate higher levels of HA when cultured \textit{ex vivo}. If this phenomenon were purely a result of the lung environment such as high pulmonary artery pressures, it would be expected to be lost in culture, especially when the cells have been passaged. The support of abnormal lung environment, PASMCs from both control and IPAH lungs have a similar pattern of HAS and Hyal expression, and when stimulated by poly I:C, PASMCs from IPAH and control lungs produce similar levels of HA.

What is it in the lung environment that can have such an effect on PASMCs? \textit{In vitro} studies show that stimulation of synthesis of HA by SMCs and endothelial cells can be in response to a variety of conditions (9) including inflammatory cytokines such as TGFβ-1 and PDGF (35, 44, 48), infectious agents (9), toxins (2, 27, 32, 41), hypoxia (15, 21, 36), or endoplasmic reticulum (ER) stress (32). Any of these factors may be responsible for the inherently high production of HA by PASMCs in IPAH. Local tissue hypoxia (real or perceived by the cells) is likely to have a major role. Whether it is systemic or local, hypoxia is a known feature of IPAH. It has also been reported that hypoxia induces HA synthesis (21). Furthermore, endogenous NO, which is known to be deficient in IPAH (18, 19, 26, 52), has an important role in hypoxia-enhanced HA synthesis (21). Hypoxia can stimulate the production of HA and the activity of hyaluronidase, which may promote angiogenesis (15) another major feature of IPAH.
Another recognized mechanism for increased production of HA by SMCs is ER stress (32). Our data, however, do not support this mechanism in IPAH PASMCs. This is based on our finding that expression of the KDEL sequence containing ER chaperone proteins Grp 78 and Grp 96 was similar in IPAH and control PASMCs. A more recently recognized mechanism for increased HA production \textit{in vitro} is the presence of high glucose concentrations in the culture media (49). This is not the case in our cells since the glucose concentrations were lower than those known to be associated with increased HA production.

So what is the role of hyaluronan in the pathobiology of IPAH? Based on our data, there are several possibilities. Hyaluronan consists of various molecular weight components \textit{in vivo} especially in disease states. HA of different molecular weights may have very different physiological effects on cellular proliferation, migration, differentiation as well as regulation of inflammation. While large molecular weight HA is thought of more as a structural, wound healing and remodeling molecule, HA with very low molecular weight has proinflammatory properties (43). Therefore, the size of HA that is upregulated in IPAH patient plasma and isolated PASMC can be helpful in understanding the potential role HA plays in IPAH.

Our finding that most of the HA in IPAH is in the very large molecular weight range (> 1 million Daltons) suggests that HA plays a major structural role in remodeling and angiogenesis in IPAH. In support of this concept, tissue staining of the plexogenic lesions in IPAH revealed widespread staining for HA surrounding the lesions, but the area of most intense staining corresponded to areas of remodeling where there is medial destruction and new collagen deposition.

Interestingly, however, our work also shows that there is a small amount of low molecular weight HA in the PASMC lysates from IPAH. Furthermore, inflammatory cells can
bind to HA produced by unstimulated PASMCs suggesting that HA produced by PASMCs in IPAH may serve a role in inflammatory cell binding to PASMCs. Since our untreated/unstimulated IPAH PASMCs constitutively express measurable amounts of HA on their surface that is also adhesive for inflammatory cells, we speculate that our cells are different, react differently to stimulation, or are induced by a different mechanism. Inflammation is a component in the pathobiology of IPAH. A significant inflammatory component is often reported in the plexiform lesions (11, 46). T cells, B cells, and macrophages seem to contribute to the inflammatory infiltrate in IPAH (11, 46). Inflammation is a common factor in the remodeling of all forms of severe and progressive IPAH (7, 46), and remodeling of the ECM in IPAH is considered to be caused by an ongoing local inflammatory process. Moreover, recent evidence indicates that CD44 on inflammatory cells can bind to HA based cables and, in this way, engage leucocytes recruited to the tissue by an inflammatory stimulus (20).

In view of evidence that HA/CD44 can activate the RhoA/Rho kinase pathway (5), and that this signaling pathway is involved in mediating sustained pulmonary vasoconstriction in experimental models of pulmonary hypertension (23, 33, 34), it could also be speculated that the increased HA is inducing pulmonary vasospasm.

Thus, HA produced by IPAH PASMCs could be involved not only in the structural changes of the remodeling process but also as a component of the cellular recruitment, interactions, inflammation and vasospasm.

Figure 11 depicts our proposed model of HA production and secretion by PASMCs from IPAH and control lungs. PASMCs from IPAH lungs spontaneously produce high levels of HA and have lower expression of HAS2 and Hyal2 compared to controls. IPAH patients have higher than normal levels of circulating HA in the blood, and PASMCs in culture secrete more HA into
the media than control PASMCs. Thus, while all smooth muscles cells can produce HA upon appropriate stimulation (9, 51), our novel data demonstrate that PASMCs from IPAH lungs produce high levels of HA without stimulation. This is an unusual and novel finding. Based on our findings in cultured cells, we believe that the source of high HA in IPAH patient plasma is likely the proliferating smooth muscle cells.

Our findings suggest that high levels of HA in patients with IPAH have an important role in the pathobiology of the disease and could serve as a biomarker of cellular proliferation and vascular remodeling. Furthermore, our findings reveal the important role of the matrix and the lung milieu in the pathobiology of IPAH and as potential targets for therapy aimed at halting or reversing cellular proliferation and vascular remodeling. Identification of the mediators that contribute to HA production and degradation could be useful in the development of new treatment or monitoring strategies in IPAH. These findings are particularly important in view of the fact that the future progress in IPAH therapy depends on our ability to target vascular remodeling in this disease.
Text footnotes

The abbreviations used are: HA, hyaluronan; IPAH, idiopathic pulmonary hypertension; PASMC, pulmonary artery smooth muscle cell; PAEC, pulmonary artery endothelial cell; SMC, smooth muscle cell; ECM, extracellular matrix; FACE, fluorophore-assisted carbohydrate electrophoresis; ER, endoplasmic reticulum; vWBF, von Willibrand Factor; H&E, hematoxylin and eosin; kDa, kilo Dalton; HiHA, high molecular weight HA; LoHA, low molecular weight HA; TGFβ1, transforming growth factor, beta-1; PDGF, platelet-derived growth factor.
Acknowledgments

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References


Figure legends

**Figure 1:** High levels of HA in IPAH. Plasma HA levels were measured by competitive binding assay in 9 healthy individuals and in 22 IPAH patients. HA levels were also measured in conditioned media of PASMCs from IPAH and control lungs. Plasma HA in IPAH patients was significantly higher compared to healthy controls (A). IPAH PASMC supernatants contained higher levels of HA than control cells (B).

**Figure 2:** Lung tissues from IPAH (left column) and control (right column) lungs were stained for HA (HA binding protein), smooth muscle cells (smooth muscle actin), and endothelia cells (von Willibrand factor) subjects. The pictures focus on small (100-200 um) pulmonary arterioles. H&E staining demonstrates the expected smooth muscle hypertrophy and hyperplasia in the IPAH arteriole (arrow in panel A) compared to control (arrow in panel B). There is more intense HA (green) staining surrounding the IPAH arteriole (C) compared to the control (D). Panels E and F are negative controls of the same sections stained by the secondary antibody only. The minimal green staining here represents autofluorescence by the elastica and the blue staining represents the DAPI staining of the nuclei. Panels G and H are composite pictures of SMC (red), HA (green), and endothelial cells (purple) for the same sections of IPAH and Control lung tissues respectively.

**Figure 3:** HA in a plexogenic IPAH lesion. Tissues were stained similar to figure 3: lungs were stained for HA (HA binding protein), smooth muscle cells (smooth muscle actin), and endothelia cells (von Willibrand factor). H&E staining in panel A demonstrates the typical features of a plexogenic lesion including focal medial sclerosis (arrowhead) with adjacent ectatic arterial segments that lead into the slit like vascular spaces (open arrow). There is also an area of medial destruction with new collagen deposition (arrow). Panel B shows the HA staining HA and panel C is the negative control section stained with the secondary antibody only. Panel D is a composite picture of SMC (red), HA (green), and
endothelial cells (purple) of the same sections. While there is widespread staining for HA (green) surrounding the lesion, the area of most intense staining seems to correlate to areas of medial destruction with new collagen deposition on the H&E staining (closed arrows in Panels A, B, and C).

**Figure 4:** Increased HA production by unstimulated IPAH PASMCs. Fluorescence light microscopic images of HA in monolayers of PASMCs from IPAH (A) and healthy control (B) lungs. Nuclei are blue (DAPI stain), and HA is green revealing the pericellular coats (arrow heads in IPAH and control cells) and extracellular cable structures (arrows in IPAH cells). IPAH cells produce distinct HA cables that are not seen in control cells. HA levels were quantified in cell lysates by FACE. Standard ladder (std) shows the different sugar components, and arrows mark HA (C). The p value for the fluorescence intensities and standard errors were calculated from four experiments each done in triplicate (D).

**Figure 5:** U937 monocytic cells bind to HA produced by IPAH PASMCs. Fluorescence light microscopic images demonstrate binding of U937 monocytic cells (blue nuclei, open arrows) to HA cables (closed arrows) produced by IPAH PASMCs (A). Healthy control PASMCs make few if any HA cables for the U937 cells to bind (B). Adhesion of radiolabeled U937 cells was much higher in IPAH than controls. This binding was HA specific as it was abrogated by the addition of the HA specific enzyme, bovine testicular hyaluronidase (BTHA-ase). poly I:C stimulation of IPAH and control cells resulted in enhanced mononuclear cell binding. This binding was also HA specific as it was abrogated by bovine testicular hyaluronidase treatment (C).

**Figure 6:** HAS and Hyal expression in IPAH and control PASMCs. RTQ-RT-PCR SYBR Green analysis was done to determine the expression of HAS1, HAS2, HAS3, Hyal1, and Hyal2 genes in both control and IPAH PASMCs. Expression values and experimental error were calculated using GAPDH as
a control for the amount of cDNA. HAS2 is the predominant HAS in both IPAH and control PASMCs and expression levels are lower in IPAH (A). Hyal2 is the predominant Hyal in both IPAH and control PASMCs, and expression levels are lower in IPAH (B).

Figure 7: Western blot analysis for Hyal2 from IPAH and control lungs. Proteins were separated by SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were probed with rabbit anti-Hyal2 antibody. Lanes 1, 2, and 3, are from control PASMCs and lanes 4, 5, and 6 are from IPAH PASMCs. Expression of Hyal2 was not different between IPAH and control PASMCs. Equal loading is demonstrated by GAPDH.

Figure 8: Zymography for Hyaluronidase. Hyaluronidase activity by Zymography of plasma (A) and PASMC supernatants (C) from IPAH and Controls. The white bands reflect HA clearance by Hyaluronidase activity. Arrow shows the size of hyaluronidase (63 kDa) in Control (lanes 1, 2, 3) and IPAH (lanes 5, 6, 7). No sample was loaded in lane 4 as a negative control. The number on the left side represents molecular mass markers in kDa. Fluorescence intensities for plasma (B) and PASMC culture supernatant (D) were calculated by Image J program as described under materials and methods. The p value for the fluorescence intensities and standard errors were calculated from four experiments each done in triplicate. The hyaluronidase activity is significantly lower in plasma and PASMC supernatants from IPAH compared to controls.

Figure 9: HA sizing gel. IPAH (lanes 4 and 5) and control (lanes 6 and 7) PASMC lysates were loaded on 1% agarose gel. Samples in lanes 2 and 4 had hyaluronidase added as negative controls. High, low and Mega HA markers were loaded in lanes 1-3 as labelled. HA in both IPAH and control PASMCs (arrows) is predominantly of very high molecular weight in the range of 3050 to 4570 kDa (bracket 1). In IPAH, there is also another less intense band in the range of 110 to 214 kDa range (bracket 2).
Figure 10: Western blot analysis for KDEL sequence containing proteins Grp 78 and Grp 96 in PASMCs. PASMCs cell lysate from IPAH and control lungs were separated by SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were probed with mouse anti-KDEL antibody. The locations of Grp 96 the Grp78 as well as actin are indicated by arrows. Lanes 1, 3, and 5 are from IPAH PASMCs and lanes 2, 4, and 6 are from control PASMCs. Expression of the KDEL sequence containing proteins Grp 78 and Grp 96 was not different between IPAH and control PASMCs. Equal loading is demonstrated by beta actin.

Figure 11: Model of HA production and secretion by PASMCs from IPAH and control lungs. PASMCs from IPAH lungs spontaneously produce high levels of HA and have lower expression of HAS2 and Hyal2 compared to controls. IPAH patients have higher than normal levels of circulating HA in the blood, and PASMCs in culture secrete more HA into the media than control PASMCs.
### Table 1: Demographics and clinical characteristics of IPAH study subjects

<table>
<thead>
<tr>
<th>Subjects with IPAH</th>
<th></th>
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<tbody>
<tr>
<td>n</td>
<td>22</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>46±3.3</td>
</tr>
<tr>
<td>Sex, F/M</td>
<td>21/1</td>
</tr>
<tr>
<td>FEV₁ (liters)</td>
<td>2.4±0.2</td>
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<tr>
<td>FEV₁ (% of predicted)</td>
<td>82.9±4.4</td>
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<tr>
<td>FVC (liters)</td>
<td>3.1±0.2</td>
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<tr>
<td>FVC (% of predicted)</td>
<td>91.5±3.8</td>
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<tr>
<td>MPAP (mmHg)</td>
<td>94.7±2.7</td>
</tr>
<tr>
<td>PVR (woods units)</td>
<td>12.7±1.7</td>
</tr>
<tr>
<td>CI (liters/min/m²)</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>AST (mg/dl)</td>
<td>281±144</td>
</tr>
<tr>
<td>ALT (mg/dl)</td>
<td>121±58</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.0±0.1</td>
</tr>
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</table>

**Medications:**

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<th>Number of patients</th>
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<tr>
<td>Oxygen</td>
</tr>
<tr>
<td>Coumadin</td>
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<tr>
<td>Diuretic</td>
</tr>
<tr>
<td>Digoxin</td>
</tr>
<tr>
<td>Calcium channel Blocker</td>
</tr>
<tr>
<td>Prostanoid</td>
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<tr>
<td>Endothelin blocker</td>
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<td>Phosphodiesterase inhibitor</td>
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### Table 2: List of primers used in RTQ-RT-PCR.

<table>
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<th>Primers*</th>
<th>Sequence</th>
<th>Gen Bank Accession no.</th>
<th>Nucleotide position</th>
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<tr>
<td>hHAS1 fwd</td>
<td>CCT GCA TCA GCG GTC CTC TA</td>
<td>NM_001523</td>
<td>943-962</td>
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<tr>
<td>hHAS1 rev</td>
<td>GCC GGT CA-T CCC CAA AAG 3</td>
<td>NM_001523</td>
<td>1056-1039</td>
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<tr>
<td>hHAS2 fwd</td>
<td>CGC AAC ACG TAA CGC AAT TGG</td>
<td>NM_005328</td>
<td>1038-1058</td>
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<tr>
<td>hHAS2 rev</td>
<td>CCA CAG ATG AGG CTG GGT CAA G</td>
<td>NM_005328</td>
<td>1184-1205</td>
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<tr>
<td>hHAS3 fwd</td>
<td>GGC GAT TCG GTG GAC TAC ATC C</td>
<td>AF232772</td>
<td>769-790</td>
</tr>
<tr>
<td>hHAS3 rev</td>
<td>ACG CTG CTC AGG AAG GAA ATC C</td>
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<td>917-938</td>
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<tr>
<td>hHyal-1 fwd</td>
<td>GTA TGT GCA ACA CCG TGT GGC</td>
<td>HSU96078</td>
<td>1390-1410</td>
</tr>
<tr>
<td>hHyal-1 rev</td>
<td>CAG GCG TGA GCT GGA TGG AGA</td>
<td>HSU96078</td>
<td>1790-1770</td>
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<tr>
<td>hHyal-2 fwd</td>
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<td>1004-1025</td>
</tr>
<tr>
<td>hHyal-2 rev</td>
<td>TCA CCC CAG AGG ATG ACA CCA G</td>
<td>BC000692</td>
<td>1138-1117</td>
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<tr>
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<td>ACC ACA GTC CAT GCC ATC AC</td>
<td>X54989</td>
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<tr>
<td>hGAPDH rev</td>
<td>TCC ACC ACC CTG TTG CTG TA</td>
<td>X54989</td>
<td>2456-2481</td>
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</table>

*Shown are the names, sequences, source sequence accession numbers, and relative positions of the primers used for RTQ-RT-PCR. The annealing temperature for all RTQ-RT-PCR primers was 65 °C.*

*The letter h in front of primer names designates primers specific for human genes. fwd, forward primer; rev, reverse primer*
Figure 1

A

HA in Plasma (ng/mL)

p=0.02

0 50 100 150 200 250 300 350 400

Control IPAH

B

HA in Media ug/mL

p=0.04

0 5 10 15

Control IPAH
Figure 2
Figure 4

A. Fluorescence intensity for Control and IPAH samples.

B. Magnified view showing specific cellular structures.

C. Gel electrophoresis showing Std, Control, and IPAH bands.

D. Bar graph comparing fluorescence intensity between Control and IPAH samples, with a significance level of p=0.001.
Figure 5

Panel A: An image showing cellular structures with arrows indicating specific regions.

Panel B: Another image with a similar cellular structure.

Panel C: A bar graph comparing U937 cells bound (x10⁴) under different conditions:
- No treatment
- BTHA-ase
- poly I:C
- poly I:C and BTHA-ase

The graph includes p-values for each comparison:
- No treatment vs. poly I:C: p=0.01
- No treatment vs. poly I:C and BTHA-ase: p=0.47
- Poly I:C vs. poly I:C and BTHA-ase: p=0.43
- Poly I:C and BTHA-ase vs. BTHA-ase: p=0.65
Figure 6

A

Expression levels

p = 0.0004

Control PASMC IPAH PASMC

HAS1 HAS2 HAS3

B

Expression level

p = 0.008

Control PASMC IPAH PASMC

Hyal1 Hyal2

Figure 6

Expression levels

p = 0.0004

Control PASMC IPAH PASMC

HAS1 HAS2 HAS3

Hyal1 Hyal2
Figure 7

Hyal2 67 kDa

GAPDH 38 kDa
Figure 8

A

B

C

D

Fluorescence Intensity

Fluorescence Intensity

Fluorescence Intensity

Fluorescence Intensity

Control    IPAH
1     2     3    4      5     6     7
100  75  60  37  25

Control    IPAH
1     2     3      4      5      6    7
100  75  60  37  25

p=0.04

p=0.04
Figure 9

[Image of a gel electrophoresis with molecular weight markers and labeled lanes for Hi Mw, Lo Mw, Mega, IPAH, IPAH+ HAase, Ctrl, and Ctrl+ HAase. The gel shows bands at various molecular weights, including 6100 kDa, 4570 kDa, 3050 kDa, 1520 kDa, 1510 kDa, 1090 kDa, 966 kDa, 572 kDa, 495 kDa, 310 kDa, 214 kDa, and 110 kDa.]

- Lane 1: Bands at 6100 kDa and 4570 kDa
- Lane 2: Bands at 3050 kDa and 1520 kDa

Areas labeled 1 and 2 indicate specific bands or regions of interest.
Figure 10

Grp96
Grp78
β-Actin

130 kDa
95 kDa
72 kDa
50 kDa
37 kDa
Figure 11