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Significance of the 2-*O*-sulfo group of L-iduronic acid residues in heparin on the growth inhibition of bovine pulmonary artery smooth muscle cells

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ABSTRACT

Heparin inhibits the growth of several cell types in vitro, including bovine pulmonary artery smooth muscle cells (BPASMCs). To understand more about the heparin structure required for endogenous activity, chemically modified derivatives of native heparin and glycol-split heparin, namely, 2-O-desulfonated iduronic/glucuronic acid residues in heparin, and 2-O-desulfonated iduronic residues in glycol-split heparin were prepared. These were assayed for their antiproliferative potency on cultured BPASMCs. All of the 2-O-desulfonated heparin derivatives had significantly decreased less antiproliferative activity on BPASMCs. These results suggest that the 2-*O*-sulfo group of iduronic acid residues in heparin's major sequence is essential for the antiproliferative properties of heparin. The size of heparin does not affect the growth-inhibitory properties of heparin on BPASMCs at the three dose levels examined.

1. Introduction

Heparin (HP) (Fig. 1A) is found mostly in the mast cells of a wide variety of species. HP is composed of repeating residues of alternating hexuronic acid, that is, glucuronic (GlcA)/iduronic (IdoA) acid, and glucosamine (GlcN) residues with a (1→4) linkage. Sulfonate group substitution is observed on the hydroxyl groups at C-2 of GlcA/IdoA and at C-4 and C-6 of the GlcN residues. GlcN residues are also modified with sulfo and acetyl groups on the C-2 amino group. The ratio of *N*-sulfo to *N*-acetyl GlcN residues is approximately 4:1 in HP.[1]

Smooth muscle cells (SMCs) play an important role in pulmonary hypertension associated with chronic hypoxia. The proliferation of SMCs is proposed to be a key process in increasing pulmonary hypertension.[2–5] HP inhibits bovine pulmonary artery SMC's proliferation both in vivo and in vitro.[6–10] The structural features of HP responsible for this antiproliferative activity include its ionic character in addition to other poorly understood factors. For the last several years our laboratory has been involved in an in vitro study to determine the structural capacity of HP to inhibit the proliferation of bovine pulmonary artery SMCs.[11] In earlier studies we found that: (a) HPs antiproliferative properties reside in the glycosaminoglycan (GAG) chain;[12] (b) both *N*-sulfo groups and *N*-acetyl groups are required for an antiproliferative effect;[13] (c) fully O-sulfonated HP did not exhibit enhanced antiproliferative activity over that of native HP;[14] and (d) the GlcN residues in HP are not critical as GAGs containing galactosamine also show activity[14]; (e) loss of *N*-sulfo and 6-*O*-sulfo groups in the GlcN residues of HP reduces antiproliferative potency.[15] Wright et al.[16] have shown that 2-*O*-sulfo groups in uronic acid residues in the HP hexasaccharide fragment are not required for antiproliferative properties of SMC's. In contrast, Tiozzo et al.[17] found that increasing 2-O-desulfonation in HP decreases antiproliferative activity. The same group also found that very low-molecular-weight HP about 2.5 kD had negligible antiproliferative activity. Recently, Kanabar et al.[18] reported that antiproliferative activity requiring an HP chain size of at least a 3 kD.

The purpose of this investigation is to assess whether *O*-sulfo groups at C-2- of uronic acid, that is, IdoA 2S, present in the major sequence of HP (Fig. 1A), are important for the antiproliferative effect of HP on bovine pulmonary artery (BPA) SMCs. In addition, we investigate whether the size of HP is critical for the antiproliferative activity of HP on BPASMCs.

2. Experimental

2.1. Materials

Porcine mucosal HP and low-molecular-weight HP sodium salts H3393, H8537, and H3400 were obtained from Sigma Chemical Co.

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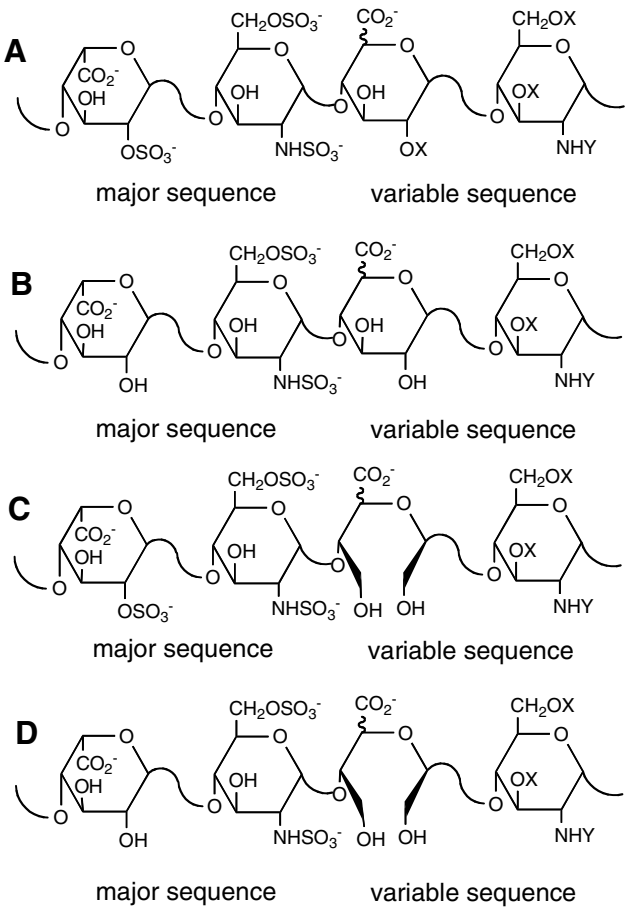

Figure 1. (A) Major and variable sequences of repeating disaccharide units in HP. (B) Major and variable sequences repeating disaccharide units in chemically modified HPs obtained by 2-O-desulfonation. (C) Major and variable sequence repeating disaccharide units in glycol-split HP derivatives obtained by periodate oxidation, followed by $NaBH_4$ reduction. (D) Major and variable sequence repeating disaccharide units in 2-O-desulfonated glycol-split HP derivatives; X = H or SO_3^-; Y = Ac and SO_3^-.

(St. Louis, MO) having an average molecular weight 16.5, 5.0, and 3.0 kD, respectively. Fetal bovine serum was obtained from Bio Whittaker (Walkersville, MD). Cell culture medium, RPMI-1640 (Mediatech, Washington, DC), contained streptomycin (Lilly, Indianapolis, IN), penicillin (Pfizer, New York, NY), and amphotericin B (GIBCO, Grand Island, NY), which were purchased commercially. All chemicals for modifying HP were obtained from Aldrich Chemical Co.

2.2. Methods

2.2.1. 2-O-Desulfonation of different size HPs

These were prepared by a known method.[19] Briefly, each HP sample (200 mg) was dissolved in 0.2 M NaOH (50 mL) and lyophilized. Water (10 mL) was added, the yellow solution was neutralized with HCl, and the solutions were dialyzed against water using Sigma benzoylated dialysis tubing MWCO 1000 before being lyophilized to obtain an off-white powder.

2.2.2. Preparation of glycol-split derivatives of different size HP by periodate oxidation followed by $NaBH_4$ reduction

These derivatives were prepared by known methods.[20] Briefly, each HP sample (200 mg) was dissolved in water (5 mL) and 0.2 M sodium periodate (5 mL) added, and the solution was stirred in the dark at 4 °C for 24 h. Ethylene glycol (2 mL) was then added, and the solution was stirred an additional 4 h. The solution was dialyzed for 6 h as before against water (6 × 1 h changes) and sodium borohydride (100 mg) was then added with stirring at room temperature for 3 h. The solution was neutralized with acetic acid and dialyzed as above. Glycol-split HP derivatives were treated with sodium hydroxide, as described in Section 2.2.1, was used to obtain 2-O-desulfonated glycol-split HP derivatives.

2.2.3. Preparation of 2-desulfonated glycol-split HP derivatives

The glycol-split HP derivatives prepared in Section 2.2.2 were treated with sodium hydroxide as described in Section 2.2.1 to obtain the glycol-split derivatives of 2-O-desulfonated HP.

2.2.4. Characterization of HPs and HP derivatives

The molecular weight of each sample was analyzed using polyacrylamide gel electrophoresis (PAGE),[21] and their chemical structures were determined using ^1H NMR spectroscopy.[22]

HP and its derivatives were analyzed using PAGE (Mini-gel apparatus, Bio-Rad, Hercules, CA.) An equal amount of each sample (5 μL at 2 mg/mL) was combined with one volume of 50% (w/v) sucrose, and the mixture was loaded into a stacking gel of 5% (total acrylamide) and fractionated with a 22% resolving gel. Electrophoresis was performed at 200 V for 80 min. The gel was stained and fixed with Alcian Blue in 2% (v/v) acetic acid. The average molecular weights of these samples were determined using a standard curve based on heparin oligosaccharide standards. HP samples (10 mg) were dissolved in 0.5 mL of D_2O (99.996%, Sigma) and freeze-dried three-times to remove the exchangeable protons. The samples were re-dissolved in 0.5 mL of D_2O. Spectra were recorded at 300 K on Bruker 500 MHz spectrometer.

2.2.5. Cultured bovine pulmonary artery smooth cells (BPASMCs) proliferation assay

BPASMC proliferation assays were preformed as previously described.[23,24] Briefly, isolated BPASMCs in passages 4–6 were seeded at 1.5×10^4 cells/well into 6-well tissue culture plates, grown for two days, then growth arrested for 48 h by reducing the serum concentration of the medium from 10% to 0.1%. The medium was then changed for experimental samples to contain either standard medium [RPMI-1640 with 10% fetal bovine serum (FBS)], growth arrest media (0.1% FBS) or standard media containing HPs and their chemically modified derivatives. All media contained streptomycin (10 μg/mL), penicillin (100 U/mL), and amphotericin B (1.25 μg/mL). After 4 days, BPASMCs present in the cell culture wells were rinsed with Hank's balanced salt solution to remove the remaining cell culture medium. No dead cells (trypan blue exclusion) were observed in either the control preparations or in the heparin derivative-treated culture media. After detachment of BPASMCs with trypsin/EDTA, the cell numbers were determined by direct cell count.

The HP derivative was dissolved (1 mg/mL) in distilled sterile water, and 14 μL, 140 μL, or 1.4 mL of this solution was added to the culture medium (13.99, 13.86, and 12.6 mL, respectively). Then 2 mL of the resulting media was added to each well. The chemically modified HP derivatives and parent HP samples were completely soluble, as the culture medium was clear (no turbidity) after addition of the HP derivatives. In the present study 1 μg/mL, 10 μg/mL, and 100 μg/mL of HP derivatives were used.

The percent growth inhibition was calculated as follows:

$$\{1 - (\text{net cell growth in treated medium}/\text{net cell growth inright standard medium})\} \times 100,$$

where net cell growth corresponds to cell growth in the standard or treated medium minus cell growth in growth arrest media. Differences in growth reflected in the differences in thymidine incorporation were not measured because the secretion of high amounts of endogenous thymidine in cell culture prevents the assessment of DNA synthesis with labeled thymidine.[25]

2.2.6. Cell-growth statistics

Results are reported as mean ± standard error of the mean. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the STATVIEW software package (SAS Institute, Cary, NC 27513) for Macintosh computers. If ANOVA were significant, multiple comparisons were made using the Fisher protected least significant difference (PSLD) test. Significance was set as $p < 0.01$.

3. Results and discussion

We have prepared chemically modified 2-O-desulfonated HP derivatives to evaluate whether the 2-*O*-sulfo groups of uronic acid residues present in the major and variable sequences of heparin (Fig. 1A) are responsible for growth inhibition of BPASMCs. These HP derivatives were obtained by treatment of HPs with sodium hydroxide to give their 2-O-desulfonated derivatives (Fig. 1B and D). The average molecular weight of the HPs and HP derivatives were examined by PAGE.[21] PAGE analyses of the 16.5-kD HP sample and its derivatives are shown in Figure 2. Analysis of this gel[21] showed the commercial HP sample labeled MW 16.5, its desulfonation, glyco-spliting, and desulfonation followed by glyco-splitting derivatives had average molecular weights of 12, 11, 12, and 6.5 kD, respectively. Similarly, the samples 5 kD and 3 kD also showed the expected reductions in molecular weights.

Each HP and HP derivative was next examined by ^{1}H NMR spectroscopy to confirm its chemical structure.[26] The ^{1}H NMR spectra of the 16.5-kD HP sample and its derivatives are shown in Figure 3. The starting HP was consistent with a pure porcine intestinal heparin[22] allowing the convenient assignment of the 12 major signals (Fig. 3A, a–l).

In each 2-O-desulfonation product IdoAS is converted to IdoA. This is clearly demonstrated by a change in the chemical shift in the H-1 signal of the iduronic acid residue from 5.1 to 4.9 (peaks b and b′) and the change in chemical shift of H-2 signal of the iduronic acid residue shifts by 0.3 ppm, from 4.15 to 3.85 (peaks d and d′) (Fig. 3A–B and C–D). In each glycol-split product the C2–C3 bond in GlcA is cleaved resulting in a shift in the H-2, and H-3 peaks of GlcA (j) causing them to shift under peak k (Fig. 3A–C and B–D). Thus, the ^{1}H NMR spectra of the derivatives are consistent with the chemical structures shown in Figure 1.

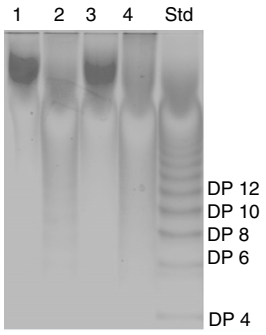

Figure 2. PAGE analysis of 16.5-kD HP and its derivatives. Lane 1: HP, Lane 2: desulfonated HP, Lane 3: glyco-split HP, Lane 4: desulfonated and glyco-split HP. Heparin-derived oligosaccharide standards are used to determine the molecular weight of each sample corresponding to disaccharide (DP 2), tetrasaccharide (DP 4), hexasaccharide (DP 6), etc.

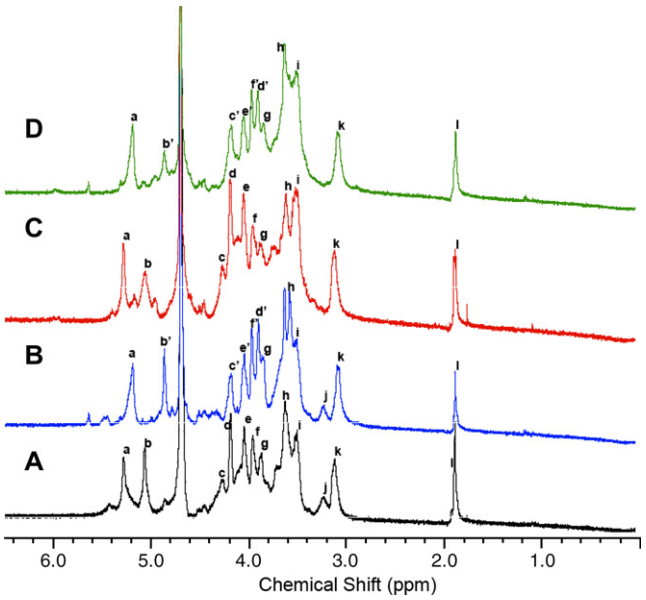

Figure 3. ^{1}H NMR spectra of 16.5-kD HP and its derivatives. (A) Starting HP, (B) desulfonated HP, (C) glyco-split HP, and (D) desulfonated and HP. Glyco-split peaks were assigned as (a) GlcNS6S, H-1; (b) IdoAS, H-1; (b′) IdoA, H-1; (c) IdoAS, H-5; (c′) IdoA, H-5; (d) IdoAS, H-2; (d′) IdoA, H-2; (e) IdoAS, H-3; (e′) IdoA, H-3; (f) IdoAS, H-4; (f′) IdoA, H-4; (g) GlcNS6S, H-5; (h) GlcNS6S, H-4; (i) GlcNS6S, H-3; (j) GlcA, H-2, H-3; (k) GlcNS6S, H-2; and (l) NAc-H. The off-scale peak at 4.7 ppm corresponds to HOD.

HPs of MW 16.5 kD, 5 kD, and 3 kD were examined as these correspond to full-length heparin (16.5 kD), low-molecular-weight heparin (5 kD) and ultra-low-molecular-weight heparin, respectively, providing a wide range of molecular weight species to assess activity. All these HP derivatives were assayed for their growth-inhibition properties against BPASMCs. Figure 4 shows the effects of 16.5 kDa and 5 kDa and their 2-O-desulfonated derivatives at three concentrations (1 μg, 10 μg, and 100 μg/mL). Cells were grown in HP, derivative-free medium was defined as 0% inhibition, and cells grown in 0.1% fetal bovine serum as 100% inhibition. Treatment of BPASMCs cultures at three dose levels of HP 16.5 kDa produced the following growth inhibition: 48.8 ± 8.8%, 66.9 ± 13.9%, 92.2 ± 5.3%, respectively. The addition of 16.5-kDa HP 2-O-desulfonated HP to the cell culture resulted in decreases in growth inhibition by 37.5 ± 6.2%, 53.3 ± 11.3%, and 78.0 ± 5.3%, respectively.

Treatment with 5-kDa HP gave a decrease in BPASMC growth inhibition of 57.7 ± 12.8%, 69.2 ± 10.6%, and 91.4 ± 7.7%, respectively, while the 5-kDa 2-O-desulfonated HP derivative afforded a decrease of 47.6 ± 10.5%, 56.9 ± 10.5%, and 76.3 ± 9.5%, respectively.

Similar decreases in BPASMC growth inhibition by 2-O-desulfonated 3-kDa HP derivative in comparison to HP (Fig. 1A and B) were also observed (Fig. 5). The percentages of cell-growth inhibition in HP and its 2-O-desulfonated derivative were 46.5 ± 6.5, 60.8 ± 7.1, 80.8 ± 10.5: 26.5 ± 12.9, 46.9 ± 8.0, and 59.1 ± 10.2, respectively. Both 2-*O*-sulfo of uronic acid residues in the major and variable regions of HP (Fig. 1A) appear to be important for the growth-inhibitory effects of HP as suggested in previous findings by Tiozzo et al.[17] This group observed that a reduction in molecular weight of HP was associated with a progressive reduction of antiproliferative activity. Our results show that the HPs size does not significantly affect its antiproliferative properties over the size range examined. These differences may result from the use of

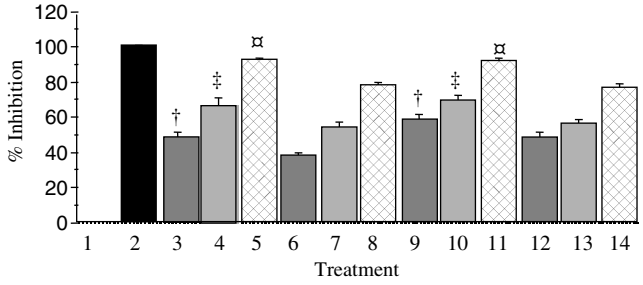

Figure 4. Inhibition of BPAMC proliferation by 16.5-kD and 5-kD 2-O-desufated HPs, including starting HPs. Percent inhibition of bovine pulmonary artery smooth muscle cell grown in media containing 10% FBS without HP as negative control (−), bar 1; 0.1% FBS without HP as positive control (+), bar 2; 10% FBS plus 1 μg of 16.5 kD HP, bar 3; 10% FBS plus 10 μg of 16.5 kD-HP, bar 4; 10% FBS plus 100 μg of 16.5-kD HP, bar 5; 10% FBS plus 1 μg of 16.5-kD 2-O-desulfonated HP, bar 6; 10% FBS plus 10 μg of 16.5- kDa 2-O-desulfonated HP, bar 7; 10% FBS plus 100 μg of 16.5-kD 2-O-desulfonated HP, bar 8; 10% FBS plus 1 μg of 5 kD-HP, bar 9; 10% FBS plus 10 μg of 5- kDa HP, bar 10; 10% FBS plus 100 μg of 5-kD HP, bar 11; 10% FBS plus 1 μg of 5- kDa 2-*O*-desulfonated HP, bar 12; 10% FBS plus 10 μg of 5-kD 2-*O*-desulfonated HP, bar 13; 10% FBS plus 100 μg of 5-kD 2-*O*-desulfonated HP, bar 14. (†) Significant inhibition of growth of BPASMCs by 1 μg of 16.5-kD HP compared to 1 μg of its 2-*O*-desulfonated derivative; (‡) significant inhibition of growth of BPASMCs by 10 μg of 16.5-kDa HP compared to 10 μg of its 2-*O*-desulfonated derivative; (¤) significant inhibition of growth of BPASMCs by 100 μg of 16.5-kD HP compared to 100 μg of its 2-*O*-desulfonated derivative; (†) significant inhibition of growth of BPASMCs by 1 μg of 5-kD HP compared to 1 μg of its 2-*O*-desulfonated derivative; (‡) significant inhibition of growth of BPASMCs by 10 μg of 5-kD HP compared to 10 μg of its 2-O-desulfonated derivative; (¤) significant inhibition of growth of BPASMCs by 100 μg of 5-kD HP compared to 100 μg of its 2-*O*-desulfonated derivative.

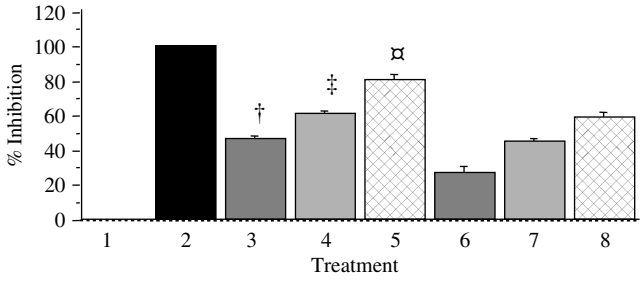

Figure 5. Inhibition of BPAMC proliferation by 3-kD 2-O-desulfonated HP, including starting HP. Percent inhibition of bovine pulmonary artery smooth muscle cell grown in media containing 10% FBS without HP as negative control (−), bar 1; 0.1% FBS without HP as positive control (+), bar 2; 10% FBS plus 1 μg of 3-kD HP, bar 3; 10% FBS plus 10 μg of 3-kD HP, bar 4; 10% FBS plus 100 μg of 3-kD HP, bar 5; 10% FBS plus 1 μg of 3-kD 2-O-desulfonated HP, bar 6; 10% FBS plus 10 μg of 3-kD 2-O-desulfonated HP, bar 7; 10% FBS plus 100 μg of 3-kD 2-O-desulfonated HP, bar 8. (†) Significant inhibition of growth of BPASMCs by 1 μg of 3- HP compared to 1 μg of its 2-O-desulfonated derivative; (‡) significant inhibition of growth of BPASMCs by 10 μg of 3-kD HP compared to 10 μg of its 2-O-desulfonated derivative; (¤) significant inhibition of growth of BPASMCs by 100 μg of 3-kDa HP compared to 100 μg of its 2-O-desulfonated derivative.

different cell types or from the different range of heparin sizes examined.

Glycol-split derivatives of 16.5 and 5-kDa HP were prepared to determine the significance of the 2-*O*-sulfo groups of the iduronic acid residues in the HP major sequence (Fig. 1A). Periodate oxidation of HP cleaved the C2–C3 bonds of the unsulfated glucuronic and iduronic acid residues, affording a ring-opened residue containing aldehyde groups at C2 and C3. Sodium borohydride reduction of the residual aldehyde functional groups to the less reactive alcohol groups present in the glycol-split HPs (Fig. 1C). These HP derivatives were 2-O-desulfonated (Fig. 1D) using the same conditions as those applied to the parent HP samples. The resulting

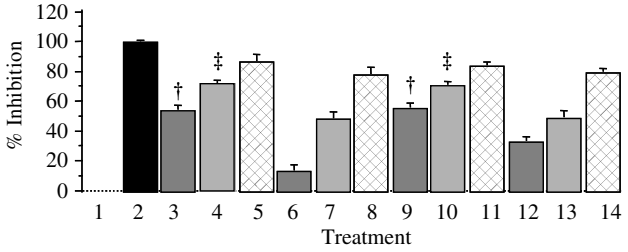

Figure 6. Inhibition of BPAMC proliferation by 16.5-kD and 5-kD 2-O-desulfonated glycol-split HPs, including starting glycol-split HPs. Percent inhibition of bovine pulmonary artery smooth muscle cell grown in media containing 10% FBS without HP as negative control (−), bar 1; 0.1% FBS without HP as positive control (+), bar 2; 10% FBS plus 1 μg of 16.5-kD glycol-split HP, bar 3; 10% FBS plus 10 μg of 16.5-kD glycol-split HP, bar 4; 10% FBS plus 100 μg of 16.5-kD glycol-split HP, bar 5; 10% FBS plus 1 μg of 16.5-kD 2-O-desulfonated glycol-split HP, bar 6; 10% FBS plus 10 μg of 16.5-kD glycol-split 2-O-desulfonated HP, bar 7; 10% FBS plus 100 μg of 16.5-kD 2-O-desulfonated glycol-split HP, bar 8; 10% FBS plus 1 μg of 5-kD glycol-split HP, bar 9; 10% FBS plus 10 μg of 5 kD glycol-split HP, bar 10; 10% FBS plus 100 μg of 5-kD glycol-split HP, bar 11; 10% FBS plus 1 μg of 5-kD 2-O-desulfonated glycol-split HP, bar 12; 10% FBS plus 10 μg of 5-kD 2-O-desulfonated glycol-split HP, bar 13; 10% FBS plus 100 μg of 5-kD 2-O-desulfonated glycol-split HP, bar 14. (†) Significant inhibition of growth of BPASMCs by 1 μg of 16.5-kD glycol-split HP compared to 1 μg of its 2-*O*-desulfonated derivative; (‡) significant inhibition of growth of BPASMCs by 10 μg of 16.5-kD glycol-split HP compared to 10 μg of its 2-O-desulfonated derivative; (¤) significant inhibition of growth of BPASMCs by 100 μg of 16.5-kD glycol-split HP compared to 100 μg of its 2-O-desulfonated derivative; (†) significant inhibition of growth of BPASMCs by 1 μg of 5-kD glycol-split HP compared to 1 μg of its 2-O-desulfonated derivative; (‡) significant inhibition of growth of BPASMCs by 10 μg of 5-kD glycol-split HP compared to 10 μg of its 2-O-desulfonated derivative.

derivatives were then assayed for their growth-inhibition properties (Fig. 6). The results of 16.5-kDa glycol-split HP and its 2-O-desulfonated derivatives produced decreases in cell growth: 54.2 ± 10.2%, 71.7 ± 7.2%, 87.1 ± 13.0%: 13.3 ± 11.5%, 48.9 ± 11.0%, and 78.8 ± 11.1%, respectively. Treatment of 5-kDa glycol-split HP and its 2-O-desulfonated derivative produced decrease in growth inhibition of bovine pulmonary SMCs of 56.1 ± 8.0%, 74.1 ± 7.9%, 84.2 ± 7.5%; 33.2 ± 9.8%, 49.6 ± 11.4%, and 79.9 ± 6.2%, respectively. There were significant differences at both 1 μg/mL and 10 μg/mL.

In conclusion, we have clearly demonstrated that the presence of 2-*O*-sulfo groups on the iduronic acid residues in the major sequence of HP is essential for BPASMC antiproliferative activity. The size of the HP preparations, from 3 to 16.5 kDa, does not affect their growth-inhibitory properties. These results may lead us to design effective candidates for treatment of pulmonary hypertension where the major problem is excess pulmonary SMCs growth.

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