**Background**—Idiopathic pulmonary arterial hypertension (IPAH) is a cardiopulmonary disease characterized by cellular proliferation and vascular remodeling. A more recently recognized characteristic of the disease is the dysregulation of glucose metabolism. The primary link between altered glucose metabolism and cell proliferation in IPAH has not been elucidated. We aimed to determine the relationship between glucose metabolism and smooth muscle cell proliferation in IPAH.

**Methods and Results**—Human IPAH and control patient lung tissues and pulmonary artery smooth muscle cells (PASMCs) were used to analyze a specific pathway of glucose metabolism, the hexosamine biosynthetic pathway. We measured the levels of \(O\)-linked \(\beta\)-N-acetylglucosamine modification, \(O\)-linked \(\beta\)-N-acetylglucosamine transferase (OGT), and \(O\)-linked \(\beta\)-N-acetylglucosamine hydrolase in control and IPAH cells and tissues. Our data suggest that the activation of the hexosamine biosynthetic pathway directly increased OGT levels and activity, triggering changes in glycosylation and PASMC proliferation. Partial knockdown of OGT in IPAH PASMCs resulted in reduced global \(O\)-linked \(\beta\)-N-acetylglucosamine modification levels and abrogated PASMC proliferation. The increased proliferation observed in IPAH PASMCs was directly impacted by proteolytic activation of the cell cycle regulator, host cell factor-1.

**Conclusions**—Our data demonstrate that hexosamine biosynthetic pathway flux is increased in IPAH and drives OGT-facilitated PASMC proliferation through specific proteolysis and direct activation of host cell factor-1. These findings establish a novel regulatory role for OGT in IPAH, shed a new light on our understanding of the disease pathobiology, and provide opportunities to design novel therapeutic strategies for IPAH. (Circulation. 2015;131:1260-1268. DOI: 10.1161/CIRCULATIONAHA.114.013878.)

**Key Words:** biosynthetic pathways ■ hexosamines ■ host cell factor C1 ■ pulmonary hypertension ■ smooth muscle ■ UDP-N-acetylglucosamine-peptide beta-N-acetylglucosaminytransferase

Idiopathic pulmonary arterial hypertension (IPAH) is a rapidly progressive disease with poor prognosis that affects the heart and lungs.1–4 Presently, IPAH is considered a vasculopathy that results from the structural and morphological changes of the vasculature within the lung. Altered metabolic functions, including dysregulated glucose uptake/metabolism have been described in IPAH.5–7 and have the potential to impact several key processes implicated in the pathogenesis of the disease, including cellular proliferation. Understanding the molecular mechanisms and pathways that link dysregulation in glucose metabolism to cell proliferation can lead to a better understanding of the disease and the development of novel IPAH therapies.

**Clinical Perspective on p 1268**

The hexosamine biosynthetic pathway (HBP) serves as a sensor for metabolic flux and is a precursor for glycosylation pathways.8–10 Once activated, the HBP generates the sugar nucleotide UDP-N-acetylglucosamine (UDP-GlcNAc), which is a substrate for hyaluronan (HA) and CMP-sialic acid synthesis, and for the \(O\)-linked \(\beta\)-N-acetylglucosamine (O-GlcNAc) modification of proteins.11 \(O\)-GlcNAc modification is a cellular process similar, and often reciprocal, to protein phosphorylation with occupation of the same serine/threonine residues when the phosphate is removed.12 Typically, \(O\)-GlcNAc modification has an inverse functional relationship with phosphorylation and is important in the regulation of the target protein’s function.13

In the presence of UDP-GlcNAc, transfer of the GlcNAc moiety to serine and threonine residues within proteins is governed by the \(O\)-GlcNAc transferase (OGT).14,15 Conversely, the removal of the residue is governed by the cytosolic or nuclear \(\beta\)-N-acetylglucosaminidase (O-GlcNAc hydrolase, OGA).16,17 Recently, OGT was recognized for its dual role in the hyper \(O\)-GlcNAc modification and subsequent proteolytic activation of the cell cycle master regulator, host cell factor-1.
(HCF-1), indicating a direct role for OGT in cell proliferation.\textsuperscript{18,19} Indeed, OGT synergizes with the HBP to regulate both nuclear and cytoplasmic protein functions through the O-GlcNAc modification.

We previously demonstrated increased levels of HA in plasma and tissues in IPAH patients,\textsuperscript{20} suggesting augmented glucose flux through the HBP to increase UDP-GlcNAc, a substrate for HA synthesis. However, the functional role of the HBP and the underlying mechanisms of IPAH pulmonary artery smooth muscle cell (PASMC) proliferation have not been determined. We hypothesized that IPAH is characterized by increased HBP flux and OGT upregulation, which promotes and perpetuates PASMC proliferation. We report here that HBP flux is indeed enhanced in IPAH, and OGT levels and activity are increased. Together, these changes promote protein glycosylation leading to PASMC proliferation. The increased proliferation observed in IPAH PASMCs is directly impacted by the OGT activation of HCF-1, a phenomenon recently described in proliferating cancer cells.\textsuperscript{18} These new insights into the mechanism of PASMC proliferation can lead to the identification of novel therapeutic targets in IPAH.

Materials and Methods

Lung Tissue, PASMC Isolation, and Culture Conditions

All explanted lungs were collected either at the Cleveland Clinic through an institutional review board–approved protocol or they were provided by Baylor, Stanford, Vanderbilt, University of Alabama, and Allegheny College of Mary land under the Pulmonary Hypertension Breakthrough Initiative. Funding for the Pulmonary Hypertension Breakthrough Initiative was provided by the Cardiovascular Medical Research and Education Fund. Human lung tissues used in this study were from 8 donor lung explants not suitable for lung transplantation and 8 IPAH patients (Table 1). Human PASMCs and pulmonary arterial endothelial cells were isolated from elastic pulmonary arteries dissected from both control and IPAH lungs obtained at explantation (Table 1) by using a previously described method.\textsuperscript{21} In brief, human pulmonary arteries were minced and digested overnight in Hanks balanced salt solution containing collagenase and DNase and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Sigma, St. Louis, MO). On the removal of the pulmonary arterial endothelial cells, smooth muscle cells were released from the artery tissue, filtered with a 100-μm-pore nylon cell strainer (BD Falcon, Bedford, MA), cultured in Dulbecco modified Eagle medium/nutrient mixture-p-methyl glutamine (Sigma, St. Louis, MO) + Thiamet G (25 nmol/L, Sigma) added to block inhibition of the enzyme pyruvate dehydrogenase (Sigma/Aldrich, St. Louis, MO).

Immunohistochemistry, Immunofluorescence, and Hematoxylin and Eosin Stains

For additional methods, see Materials and Methods in the online-only Data Supplement.

Sample Preparation and Western Blotting

Frozen human lung tissue\textsuperscript{22} and PASMCs\textsuperscript{22} were prepared as previously described with O-(2-acetamido-2-deoxy-d-glucopyranosylamine) N-phenylcarbamate (50 μmol/L; Sigma, St. Louis, MO) + Thiamet G (25 μmol/L; Sigma) added to block removal of the O-GlcNAc modification and subjected to Western blot analysis. Nitrocellulose membranes were probed with antisera for the following: (1) O-GlcNAc (1:1000; clone CTD 110.6, generous gift from the laboratory of Dr Gerald Hart) and HCF-1 (1:1000; Abcam, Cambridge, MA) probed blots were developed by using enhanced chemiluminescence (Amersham, Pittsburgh, PA), and (2) blots probed for glucose:fructose-6-phosphate aminotransferase-1 (1:2000; Abcam, Cambridge, MA), OGT (1:5000, Hart Laboratory), OGA (1:5000 Hart Laboratory), β-actin (1:10000; Santa Cruz, CA), glucose transporter 1 (GLUT1; 1:2000; Millipore, Billerica, MA) and glucose transporter 4 (GLUT4; 1:2000; Millipore) were blocked, washed, and imaged by using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Fluorophore-Assisted Carbohydrate Electrophoresis UDP-Sugar Analysis

PASMCs were washed, collected, and centrifuged in cold phosphate-buffered saline. Cells were fixed with 75% cold ethanol, sonicated, and subjected to fluorophore-assisted carbohydrate electrophoresis (FACe) analysis (see online-only Data Supplement Materials and Methods).

siRNA Transfection and OGT Inhibitor Experiments

PASMCs were subjected to small interfering RNA (siRNA) knockdown with a scrambled, an antisense siRNA oligonucleotide against OGT, or an OGT-specific inhibitor (see online-only Data Supplement Materials and Methods).

Cell Proliferation and Flow Cytometry Analysis

PASMCs were pulsed for 2 hours with 10 μmol/L 5-bromo-2′-deoxyuridine (Sigma, St. Louis, MO) and subjected to flow cytometric analysis (see online-only Data Supplement Materials and Methods). The gating strategy and the relevant controls for flow cytometric analysis are shown in Figure I in the online-only Data Supplement.

Study Population for Erythrocyte Collection

Patients were recruited from the Pulmonary Vascular Program at Cleveland Clinic. Blood was drawn from patients and deposited into our biorepository. Demographic and clinical characteristics of the individuals who have pulmonary arterial hypertension (PAH) are listed in Table 2. Pulmonary hypertension was confirmed by right heart catheterization. PAH categories were identified based on the

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{IPAH lung tissue, n} & 8 \\
\textbf{Age, y} & 38.3±16.0 \\
\textbf{Female, n (%)} & 4 (50.0) \\
\textbf{PAH category, n (%)} & \\
\textbf{Idiopathic PAH} & 7 (87.5) \\
\textbf{Heritable PAH} & 1 (14.3) \\
\hline
\textbf{IPAH vascular cells,* n} & 4 \\
\textbf{Age, y} & 36.3±12.3 \\
\textbf{Female, n (%)} & 4 (100) \\
\hline
\textbf{Control lung tissue, n} & 8 \\
\textbf{Age, y} & 43.3±20.0 \\
\textbf{Female, n (%)} & 2 (25.0) \\
\hline
\textbf{Control vascular cells,* n} & 4 \\
\textbf{Age, y} & 49.5±6.45 \\
\textbf{Female, n (%)} & 3 (75.0%) \\
\hline
\end{tabular}
\caption{Demographic Information for Lung Explants and Isolated Cells}
\end{table}
Table 2. Baseline Clinical Features of PAH Patients (RBC Analysis)

<table>
<thead>
<tr>
<th>PAH (n)</th>
<th>86</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>45.9±12.3</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>70 (81)</td>
</tr>
<tr>
<td>PAH category, n (%)</td>
<td>63 (73.0)</td>
</tr>
<tr>
<td>Idiopathic PAH</td>
<td>7 (8.0)</td>
</tr>
<tr>
<td>Heritable PAH</td>
<td>6 (7.0)</td>
</tr>
<tr>
<td>CHD-PAH</td>
<td>10 (11.6)</td>
</tr>
<tr>
<td>NYHA class (n=80), n (%)</td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>45 (56)</td>
</tr>
<tr>
<td>III–IV</td>
<td>35 (44)</td>
</tr>
<tr>
<td>6MWD, meters (n=85)</td>
<td>410.0±128.0</td>
</tr>
<tr>
<td>BNP, pg/mL (n=81)</td>
<td>38 (13.5–107.5)</td>
</tr>
<tr>
<td>RAP, mm Hg (n=85)</td>
<td>9.0±5.8</td>
</tr>
<tr>
<td>mPAP, mm Hg</td>
<td>52.2±14.2</td>
</tr>
<tr>
<td>CI, L·min⁻¹·m⁻² (n=80)</td>
<td>2.5±0.9</td>
</tr>
<tr>
<td>PVR, Wood units (n=83)</td>
<td>10.2±6.4</td>
</tr>
</tbody>
</table>

Data are presented as mean±standard deviation (except for BNP where the data are presented as median (25th and 75th percentile) or number (%) as appropriate. BNP indicates B-type natriuretic peptide; CHD, congenital heart disease; CTD, connective tissue disease; CI, cardiac index; 6MWD, 6-minute walk distance; mPAP, mean pulmonary artery pressure; NYHA, New York Heart Association; PAH, pulmonary arterial hypertension; PVR, pulmonary vascular resistance; RAP, right atrial pressure; and RBC, red blood cell.

Results

Glycosylation Changes in the Pulmonary Vasculature of IPAH Patients

To determine if altered glycosylation is observed in IPAH, we stained tissues from control and IPAH-explanted lungs with wheat-germ agglutinin, a lectin that specifically recognizes N-acetylglucosamine (GlcNAc) and sialic acid. IPAH tissues had an increased intensity of wheat-germ agglutinin lectin staining suggesting changes in glycosylation (Figure 1A and 1B and Figure II in the online-only Data Supplement). The increased glycosylation was much more evident in the occluded pulmonary vessels and plexogenic lesions in IPAH than in control tissues (Figure 1A through 1D). These data indicate that glycosylation changes are prominent in IPAH.

HBP Flux in IPAH

Because IPAH has increased HA levels and altered glycosylation, we examined the activation state of the HBP. As shown in Figure 1E and 1F, glutamine:fructose-6-phosphate aminotransferase-1, the HBP rate-limiting enzyme, was increased in IPAH lung tissue (control, 8.2±7.0; IPAH, 28.1±12.4, P=0.002). A similar finding was determined in IPAH PASMCs isolated from the lung explants (Figure 1G and 1H, glutamine:fructose-6-phosphate aminotransferase-1: control, 4.1±1.2; IPAH, 7.1±2.0, P=0.02). Importantly, UDP-GlcNAc pools were reduced in IPAH PASMCs in comparison with controls (Figure 1I; control, 103.7±34.0; IPAH, 46.1±17.8, P=0.04). Collectively, these data suggest that the HBP is augmented in IPAH.

Global Increase in OGT-Facilitated O-GlcNAc Modification of Proteins in Human IPAH Lungs and PASMCs

To determine the outcome of HBP flux on the dynamic post-translational modification by O-GlcNAc, we assessed the changes in global protein O-GlcNAc levels, and the levels of OGT and OGA in IPAH, as well. The global O-GlcNAc modification on proteins was increased in IPAH human lung tissue in comparison with controls (Figure 1J–1L; control, 7.05±1.6; IPAH, 13.0±2.9, P=0.001; and Figure III in the online-only Data Supplement). Consistent with the O-GlcNAc changes, OGT levels were also increased in IPAH lung tissue in comparison with controls (Figure 1E and 1F; control, 17.4±8.5; IPAH, 33.4±13.9, P=0.027) without alterations in OGA levels (Figure 1E and 1F; continuous variables were compared with the Wilcoxon rank sum test. A P value of ≤0.05 was considered as significant, except when comparisons were performed as multiple comparisons among more than 2 groups, in which case a Bonferroni correction was used to adjust for the number of primary 2-group comparisons. Given the anticipated skewed distribution of OGT levels, its associations with continuous clinical parameters were assessed by using the Spearman correlation coefficient, and its associations with categorical parameters were assessed by using the Wilcoxon test. We also investigated the association between OGT levels and clinical worsening (defined as disease-related hospitalizations, lung transplantation, or all-cause mortality) inferentially by using Cox proportional hazard models with respect to OGT in its continuous form, and descriptively using Kaplan-Meier curves. All statistical analysis was done using JMP version 10.0.0 for Microsoft Windows or R version 3.0.1 (www.R-project.org).

Statistical Analysis

Summary statistics reported for continuous variable are means±standard error of mean or median (25th, 75th percentiles), with the latter used for skewed distributions. Two-group comparisons with respect to
OGT Directs Cell Proliferation in Primary PASMCs

Next, we determined whether OGT can mediate PASMC proliferation in IPAH. We used siRNA-facilitated knockdown (KD) of OGT in IPAH PASMCs and compared with control levels. Gene silencing caused a reduction in OGT (Figure 3A; 7.14±0.33 [IPAH untreated], 2.50±0.56 [control]; P<0.05 and 7.14±0.33 [IPAH untreated], 2.64±0.66 [IPAH 60 nmol/L OGT siRNA]; P<0.05) and O-GlcNAc levels (Figure 3A) in IPAH PASMCs. The consequences of OGT KD on cell density 48 hours post-siRNA transfection are shown in Figure 3B and 3C (control, 5.33×10^5±2.10×10^4; IPAH [untreated], 9.86±10^5±3.32×10^4; IPAH [scramble], 1.02×10^6±6.97×10^4; IPAH [60 nmol/L OGT siRNA], 3.18×10^5±4.41×10^4; P<0.05, respectively). In addition, cell proliferation was assessed by cellular 5-bromo-2′-deoxyuridine incorporation, which confirmed a significant decline (Figure 3D and 3E; 32.2±3.12 [IPAH untreated], 14.7±0.59 [IPAH 60 nmol/L OGT siRNA]; P<0.05). Strikingly, IPAH PASMC proliferation was decreased to control rates after OGT KD (Figure 3D and 3E; 32.2±3.12 [IPAH untreated], 14.1±1.75 [control]; P<0.05). A reduction in PAH PASMC proliferation to control levels was also determined by using an OGT inhibitor (alloxan monohydrate), consistent with the siRNA KD to OGT (Figure V in the online-only Data Supplement). These data indicate that the reduction in OGT levels results in decreased PASMC proliferation in IPAH.

Increased OGT Activates HCF-1 in IPAH PASMCs

To determine whether increased OGT expression corresponds to enhanced activity, HCF-1 cleavage, and subsequent activation in IPAH, we reduced OGT levels by siRNA KD or its activity by administration of an OGT inhibitor (TT40) in IPAH PASMCs. Reduced OGT expression resulted in a marked decrease in HCF-1 cleavage products comparable to control, 29.1±5.0; IPAH, 33.7±9.9, (P=0.25), suggesting that the O-GlcNAc elevation is a function of increased OGT.

Immunohistochemical staining for the O-GlcNAc modification of proteins within the lung tissue validated the increased O-GlcNAc levels observed in IPAH patients (Figure 2A–2H). Higher magnification of the lung tissue revealed increased O-GlcNAc staining within the pulmonary vasculature (including plexogenic lesions) of IPAH patients in comparison with controls (Figure 2A–2H). These findings are consistent with our wheat-germ agglutinin lectin stains (Figure 1A and 1B) and immunoblot data (Figure 1J–1L), suggesting increased glycosylation events within IPAH lung tissue.

Because the O-GlcNAc modification was more prevalent in the thickened tunica media of the pulmonary arteries in IPAH, we investigated the nature of this region by using primary IPAH PASMCs isolated from explanted lungs. Western blot analysis determined that the levels of O-GlcNAc–modified proteins were also elevated in IPAH PASMCs (Figure 2I and 2J, control, 5.7±2.1; IPAH, 9.6±2.4, P=0.01) and demonstrated consistently higher levels of OGT (Figure 2I and 2K; control, 2.6±0.5; IPAH, 5.8±1.6, P<0.021) similar to the lung tissue observations (Figure 1E and 1F and 1J–1L) and IPAH pulmonary arterial endothelial cell isolates (Figure IV in the online-only Data Supplement). In addition, GLUT1 levels were elevated in the IPAH PASMCs (Figure 2I and 2J; control, 7.4±2.3; IPAH, 10.6±3.1, P<0.05) analogous to reports indicating increased glucose uptake.5,26,27 However, the insulin-sensitive transporter GLUT4 was not significantly different (Figure 2I and 2M; control, 13.1±2.9; IPAH, 14.4±2.1, P=0.56). This finding is consistent with previously published reports that described GLUT1 as the predominant transporter for glucose uptake in primary PASMCs.28 Collectively, these findings suggest that the excessive HBP flux consequentially results in increased OGT transfer of O-GlcNAc within the IPAH pulmonary vasculature.
control (Figure 4A and Figure VIA in the online-only Data Supplement). Similar results were observed on reducing OGT activity with TT40 in IPAH PASMCs (Figure 4B and Figure VIB in the online-only Data Supplement), which is analogous to the reduction in HCF-1 cleavage observed in cancer cells on OGT inhibition (Figure VII in the online-only Data Supplement).

Figure 3. OGT governs proliferation in IPAH PASMCs. A, Representative immunoblots of OGT and O-GlcNAc from control and IPAH human PASMCs are shown. OGT/β-actin was quantitated based on the densitometry determined by using ImageJ software. B, Twenty-four hours after transfection, PASMCs were trypsinized and reseeded at 150,000 cells/mL, followed by snapshot assessment of cell density at the times indicated posttransfection. C, A graph of the cell density from triplicate experiments determined at 48 hours posttransfection is shown. D and E, Flow cytometric analysis was used to determine the percentage of cellular BrdU incorporation in control and IPAH PASMCs treated for 2 hours after different gene-silencing strategies. E, Flow cytometric analysis of IPAH and control PASMCs was quantitated, and the percentage of cellular BrdU incorporation was compared with untreated IPAH. siRNA transfections were performed in triplicate experiments and calculated as described in Statistical Analysis (see Materials and Methods). D, BrdU incorporation assays are shown as a representative pseudo color side scatter (SSC) plot shown for each condition. *P<0.05. BrdU indicates 5-bromo-2′-deoxyuridine; Ctrl, control; FITC, fluorescein isothiocyanate; O-GlcNAc, O-linked β-N-acetylglucosamine; IB, immunoblot; IPAH, idiopathic pulmonary arterial hypertension; OGT, O-linked β-N-acetylglucosamine transferase; and PASMC, pulmonary artery smooth muscle cells.
Increased OGT levels have been shown to be associated with poor outcomes and disease aggressiveness in patients who have prostate cancer, suggesting that increased OGT activity contributes to the disease progression. In addition, a previous report screened multiple patient RBCs for changes in global O-GlcNAc, OGT, and OGA as potential indicators of disease. Based on these studies, we analyzed global O-GlcNAc, OGT, and OGA levels from PAH and control RBCs by Western blot. Our initial assessment demonstrated an increase in the overall levels of OGT and O-GlcNAc in PAH RBCs in comparison with control with no significant change in OGA levels (Figure 5A–5E), which is consistent with Figure 1E and 1F and 1J–1L. Because OGT levels were higher in PAH RBCs, we measured OGT levels by Western blot analysis in 86 PAH RBC patient samples and associated with clinical outcomes in these patients. Data are expressed as OGT/β-actin ratios. Table 2 shows the baseline clinical characteristics of this cohort. Inverse correlations between OGT expression and right heart stroke volume (r=-0.31, P<0.01) and the 6-minute walk distance (r=-0.21, P<0.05) were observed. OGT was higher in patient New York Heart Association functional class III to IV symptoms in comparison with New York Heart Association class I to II (median, 0.48; interquartile range, 0.35–0.58 versus 0.36; interquartile range, 0.23–0.48, respectively; P<0.05). Finally, OGT levels were associated with a higher probability to clinical worsening (Figure 5F), defined as hospitalization for PAH, lung transplantation, or death (3.71; 95% confidence interval of 1.05 to 13.2; for an OGT/β-actin ratio ≥0.396; P=0.043 for OGT in its continuous form). These data suggest that OGT may be a useful marker of disease severity in PAH.

Discussion

IPAH is a rapidly progressive cardiopulmonary disease characterized by vasoconstriction and aberrant vascular cell proliferation. Current therapies mainly target vasoconstriction. The future of IPAH therapy, however, depends on our ability to identify and target the molecular underpinnings of cell proliferation in this devastating disease. In this report, we demonstrate that PASMC proliferation, a major component in the pathobiology of IPAH, is directed by increased HBP flux coupled with enhanced OGT activity, causing proteolytic activation of HCF-1. To our knowledge, this is the first description of altered HBP flux in IPAH resulting in global O-GlcNAc modification of proteins and direct activation of HCF-1 by OGT.
IPAH shares several disease signatures with cancer, including cell proliferation and dysregulated glucose metabolism and use.11 These changes in energy production and metabolic dysregulation are hallmarks of hyperproliferative cells. These cells disproportionately use metabolites (such as glucose and glutamine) to survive in energy-starved environments. In 1924, Otto Warburg found that highly proliferative cancer cells metabolize glucose via the less energy-efficient glycolysis pathway despite the presence of oxygen.52 For almost a century, scientists have speculated on the reason for this phenomenon. The current consensus is that glycolysis provides not only energy, but also the building blocks required for cell proliferation.31

Once the proliferating cells have switched to aerobic glycolysis, they produce less ATP than cells using glucose oxidation and, in turn, more rapidly take up glucose when sufficient levels are available.34 The extensive energy requirements of highly proliferative cells often result in excess use of metabolites (namely glucose, glutamine, acetylcoenzyme A, and uridine triphosphate) through multiple pathways, including the HBP. Our studies are in agreement with this notion and suggest that the increased HBP flux observed in IPAH patients (Figures 1 and 2) is an effect of the metabolic switch to aerobic glycolysis observed in proliferating cells. Studies in cancer cells have demonstrated a similar role of HBP flux, OGT, and hyper O-GlcNAc modification of proteins involved in cancer cell proliferation.10,35,36 Our data (Figure 3) demonstrates a direct role for OGT in IPAH PASMC proliferation, which correlates with the cancer cell reports.30,35,36

As a nutrient sensor of the cell, the HBP integrates the metabolism of carbohydrates, amino acids, fat, and nucleotides for the synthesis of UDP-GlcNAc, the final product of the pathway. Recently, Wellen et al18 demonstrated that treatment of glucose-starved cells with GlcNAc (to maintain hexosamine biosynthesis) rescued cell growth, indicating the importance of the HBP in cell proliferation. The effect of the HBP on cell proliferation is most likely mediated by OGT, which uses UDP-GlcNAc for the O-GlcNAc modification of proteins. This finding is consistent with the observed low levels of UDP-GlcNAc determined in the IPAH patients (Figure 1) in our study despite the increased HBP flux and glutamine:fructose-6-phosphate aminotransferase-1 protein expression.

Modulating O-GlcNAc levels has already been shown in cancer cells to contribute to cancer cell survival, proliferation, and metastasis.30,39 OGT, through protein O-GlcNAc modification, can control many cellular events including cell cycle,12 transcription,40 signal transduction,41 nutrient sensing,30 and cell stress responses.42 Interestingly, OGT can also effect cell proliferation through a completely different and distinct mechanism. Herr and colleagues18,19 recently demonstrated that OGT specifically cleaves the precursor form of the cell cycle master regulator, HCF-1. On cleavage by OGT, the N-terminal and C-terminal subunits of HCF-1 are functionally activated40 and facilitate G1 phase cell cycle progression,43 and mitosis and cytokinesis, as well.44 In line with these findings, our data suggest that cleavage of HCF-1 is directly facilitated by OGT in IPAH (Figure 4) and represents the first evidence for this phenomenon in a disease other than cancer. Moreover, this result underscores a more direct involvement of OGT in the cell proliferation process. Although the HBP and O-GlcNAc have been implicated in pathogenesis of other noncancerous diseases including heart disease,45–47 type 2 diabetes mellitus,48,49 and insulin resistance,37,52 in addition, increased OGT levels have been shown to be associated with poor outcomes and disease persistence in patients with prostate cancer,58 suggesting that the increased O-GlcNAc protein modification contributes to the disease progression. However, these findings have not been previously reported in IPAH. We found increased HBP flux, elevated OGT, and global O-GlcNAc modification of proteins in IPAH. In particular, we observed increased OGT and O-GlcNAc levels in RBCs of PAH patients (Figure 5), which is similar to the results from the IPAH lung (Figure 1). Also, we showed that increased OGT levels in RBCs are associated with poor PAH clinical outcomes (Figure 5). These data suggest that OGT levels from RBCs may be useful to gauge the severity of PAH. More importantly, the altered OGT and O-GlcNAc levels observed in PAH RBCs and lung suggests that the metabolic changes may be a systemic phenomenon. OGT may have systemic metabolic derangements. In 1 report, Pugh et al8 demonstrated that higher hemoglobin A1c levels correlated with the 6-minute walk test for IPAH patients. Thus, it is possible that our findings may be related to or a consequence of the well-documented dysregulation of glucose metabolism in this disease. Still, more research is needed to determine the systemic nature of the dysregulated metabolism in IPAH.

Fundamentally, this report demonstrates OGT as (1) an activator of HCF-1 and (2) a regulator of cell proliferation in IPAH. These findings in IPAH, along with published findings in cancer, collectively demonstrate a direct role for OGT in linking nutrient sensing of the HBP to cell cycle progression and proliferation, which may have clinical implications for a wide array of diseases beyond IPAH, including cardiovascular disease, cancer, and diabetes mellitus.

Combining the previous reports on the characterization of HA in IPAH20,22 along with the observations described here in this report, we put forth a model whereby an activated HBP regulates PASMC proliferation in IPAH as outlined in Figure 6. This model establishes a regulatory role for OGT in IPAH, sheds a new light on our understanding of the disease pathobiology, and provides opportunities to design novel therapeutic strategies for IPAH.

**Acknowledgments**

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Figure 6. Model illustrating the functional consequences of excessive HBP flux in IPAH. In IPAH, increased HBP flux leads to increased UDP-GlcNAc production that is vastly used by OGT for the O-GlcNAc modification of proteins and for the synthesis of HA. Both of these changes have been demonstrated in the cell proliferation process. As shown here, the consumption of UDP-GlcNAc by OGT facilitates enhanced O-GlcNAc modification of proteins and HCF-1 activation, resulting in an increase in vascular cell proliferation, including PASMCs. GFAT1 indicates glutamine:fructose-6-phosphate aminotransferase-1; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; O-GlcNAc, O-linked β-N-acetylglucosamine; HBP, hexosamine biosynthetic pathway; IPAH, idiopathic pulmonary arterial hypertension; HA, hyaluronan; HCF-1, host cell factor-1; OGT, O-linked β-N-acetylglucosamine transferase; and P, phosphate.

Disclosures

None.

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**CLINICAL PERSPECTIVE**

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive disease that leads to deterioration in cardiopulmonary function and premature death. Presently, IPAH is considered a vasculopathy, and metabolic dysregulation has emerged as a major area of research in the pathobiology of the disease. Several processes may be governed by the metabolic dysfunction present in IPAH, including enhanced pulmonary vascular cell proliferation, vascular remodeling, and vasoconstriction. Even though altered glucose uptake/metabolism has been characterized in IPAH, the nature of the primary abnormalities that trigger and perpetuate these characteristics remains unclear. Furthermore, the effectiveness of the current therapies is limited as they do not specifically target the pulmonary artery smooth muscle cell proliferation or vascular remodeling. In this report, using human IPAH and control patient lung tissues and pulmonary artery smooth muscle cells, we demonstrate that the increased pulmonary arterial smooth muscle cell proliferation is directed by hexosamine biosynthetic pathway activation coupled with the enhanced of O-linked β-N-acetylglucosamine transferase/O-linked β-N-acetylglucosamine axis. Our data point to a novel molecular mechanism whereby O-linked β-N-acetylglucosamine transferase directs the proteolytic activation of host cell factor-1, causing enhanced pulmonary artery smooth muscle cell proliferation, and worse disease outcomes in IPAH. These findings establish a novel regulatory role for the hexosamine biosynthetic pathway and O-linked β-N-acetylglucosamine transferase in IPAH, shed a new light on our understanding of the disease pathobiology, and provide opportunities to design novel therapeutic strategies for IPAH. Furthermore, these findings may have clinical implications for a wide array of diseases beyond IPAH, including cardiovascular disease, cancer, and diabetes mellitus.
O-Linked β-N-Acetylglucosamine Transferase Directs Cell Proliferation in Idiopathic Pulmonary Arterial Hypertension

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SUPPLEMENTAL MATERIALS AND METHODS:

Immunohistochemistry, Immunofluorescence, and Hematoxylin and Eosin (H&E) Stains

*Immunohistochemistry*—Human control and IPAH lung sections were stained using a Ventana Benchmark XT automated immunostainer (Roche Diagnostics Corporation, Indianapolis, IN) combined with a Ventana OptiView DAB Detection kit. CC2 retrieval was performed for 32 minutes followed by Opti-View amplification (4 minutes). HQ Universal Linker and HRP Multimer dilution options were both employed. Primary antibody, mouse anti-O-GlcNAc (RL-2 antibody generous gift from Dr. Gerald Hart) was diluted 1:50 and incubated for 32 minutes with heat. Slides were counterstained with Hematoxylin II, dehydrated, cleared, and permanently mounted for viewing using the same microscope and capture software as the H&E staining.

*Immunofluorescence*—Paraffin embedded human lung tissue sections were fixed with the FLEX Signature Series reagents (Richard-Allan Scientific, Thermo Scientific, USA) and stained with a primary biotinylated WGA lectin (1:500) followed by retrieval with a streptavidin conjugated Alexa-fluor-533 antibody (1:500). Tissues were mounted with media containing DAPI overnight and sealed before images were taken. Snapshots of distinct regions were taken using a Leica DM 5500B equipped with a 20x (numerical aperture 0.4) objective. Images were taken using an attached QImaging Retiga-SRV camera and QCapture 7.0 software.

*Hematoxylin and eosin (H&E) stains*—Lung tissues taken from explanted lungs were fixed and embedded in paraffin and 4 μm sections were prepared. The sections
were stained with H&E using standard procedures for proper lung orientation and morphological assessment. Snapshots of histology were taken using a Leica DM 5500B microscope equipped with a 20x (numerical aperture 0.4) objective. Images were generated using an attached Leica DFC 425C camera and the high-performance Leica LAS software.

**Fluorophore Assisted Carbohydrate Electrophoresis (FACE) UDP-Sugar Analysis**

PASMCs were washed and collected in cold PBS scraped with a rubber policeman. PASMCs were centrifuged, pelleted and fixed with 75% cold ethanol. Lysates were generated by sonication in the 75% ethanol and kept on ice. An aliquot of lysate was taken and stored at -20 °C for DNA quantitation and normalization using the Quant-IT PicoGreen dsDNA kit (Life technologies, NY, USA). Cell debris was removed by centrifugation (16,000 × g for 10 min at 4°C). The supernatants were dried and reconstituted in 10 mM ammonium bicarbonate. The UDP-sugars were then isolated and purified using an Envi-Carb column (Sigma, St. Louis, MO, USA) procedure(1) with slight modifications. Briefly, reconstituted samples were applied to the column and washed with (i) sterile filtered water, followed by (ii) a 25% acetonitrile solution, and then (iii) a 50 mM triethylamine acetate buffer (pH 7). UDP-sugars were eluted with 2 ml of 25% acetonitrile in 50 mM triethylamine acetate buffer (pH 7) and speed-vacuumed to dryness. The UDP-sugars were prepared for AMAC conjugation by subjecting the dried sample to mild acid hydrolysis (50 mM HCl) at 100°C for 20 minutes to remove the nucleotide from the sugar monosaccharide. Acid hydrolyzed samples were then dried,
and monosaccharides were solubilized in acetic acid followed by derivatization with aminoacridine (AMAC) and sodium cyanoborohydride for FACE analysis(2). A monosaccharide standard was also run simultaneously to determine the mobility of GlcNAc-AMAC within the FACE gel. In addition, a known purified amount of UDP-GlcNAc was processed using this protocol and the percent yield was determined (data not shown). Images of the gel were captured using the G:BOX Chemi XR5 system and Gene Tools software v4.3.00. The densitometry of GlcNAc-AMAC within the FACE gel was determined using ImageJ(3) software and normalized to total cellular DNA.

**siRNA Transfection and OGT Inhibitor Experiments**

PASMCs at 85-90% confluency were transfected with either a scrambled (Ambion Applied Biosystems, Inc.; cat # AM4611, NY, USA) or an antisense siRNA oligonucleotide against OGT (Ambion Applied Biosystems, Inc.; cat # 13301, NY, USA) at 60 nM using lipofectamine 2000 (Invitrogen, USA) and incubated in Opti-MEM (Cleveland Clinic Media Core) overnight. The following day, transfection medium was changed to the SmGM-2 medium and cells were grown for an additional 24 hours. At this point, confluent PASMCs were trypsinized and re-seeded in a six-well dish at a density of 1.5x10⁵ cells/mL. PASMCs were allowed to adhere, and siRNA transfection was repeated to enrich knock-down. At 48 hours post-transfection, cells were either taken for Western blot or BrdU incorporation and flow cytometric analysis. Cell images were taken using an Olympus CKX41 microscope containing a 10x (0.25 PHP)
objective with an attached SC30 camera at 2 hours and 48 hours post-enrichment transfection.

**Cell Proliferation and Flow Cytometry Analysis**

Following a 4 hour serum starvation, PASMCs were pulsed for 2 hours with 10 μM 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO, USA) followed by trypsinization to detach cells. Subsequently, the cell suspension was centrifuged for 10 minutes (RT) at 400 x g (all centrifugation steps used these conditions). Supernatants were aspirated, and cells were subjected to ice cold 70% ethanol to a final concentration of 1 X 10^6 cells/100 μL and stored in the -80ºC freezer until all BrdU incorporation assays were finished. For preparation of flow cytometric staining, cells were thawed and washed in PBS containing 0.5% BSA, and then centrifuged and resuspended in 100 μL denaturing solution (PBS containing 0.5% Tween-20, 0.5% BSA). Following 20 minutes of incubation at room temperature, cells were washed, centrifuged, and resuspended 0.1 M sodium borate (pH 8.5) to neutralize any residual acid. Upon extensive washing, an unconjugated mouse monoclonal antibody against BrdU (1:50, Ab-3 clone BRD.3, Thermo Scientific, Waltham, MA, USA) was added to the sample and incubated at room temperature for 20 minutes followed by washes and centrifugation to remove any unbound primary antibody. The secondary FITC-conjugated goat anti-mouse (1:50, BD Pharmingen, San Diego, CA, USA) was added to the cells, incubated at room temperature for 20 minutes, and subsequently washed and centrifuged to remove unbound secondary. As a biological control, cells in parallel
experiments were prepared with no BrdU incubation or analyzed with only secondary antibody (unstained) (Supplemental Figure 2). Data were generated by flow cytometry using the LSRFortessa cell analyzer (Becton Dickinson, USA) using the standard configuration. Cells were excited at 488 nm, and the BrdU linked green fluorescence (FITC) was detected through a 515/20 bandpass filter. Flowjo software (version 10) was used for analysis and the percentage of BrdU incorporated cells was determined. The gating strategy and the relevant controls for flow cytometric analysis are shown in Supplemental Figure 1.
**SUPPLEMENTAL FIGURES:**

Supplemental Figure 1

Supplemental Figure 1. Flow cytometry control parameters. (A) Time gating was performed on a Time/SSc plot to control for fluidic disturbances. (B) Aggregates were excluded using a FSC-W/FSC-A plot. Percent BrdU positive cells were determined on a pseudo-color plot as depicted in Figure 3. As a biological control, cells were treated with (C) only secondary (unstained) or (D) no BrdU in parallel experiments.
Supplemental Figure 2. Secondary control for Control and IPAH lung tissue staining of WGA (Figure 1 A and B). Representative secondary controls for control (A) and IPAH (B) lung tissue.
Supplemental Figure 3. O-GlcNAc antibody (CTD 110.6) control for reactivity. The O-GlcNAc primary antibody was subjected to a pre-incubation with 250 mM GlcNAc in 5% BSA (GlcNAc block) for an hour at room temperature. Transferred nitrocellulose membranes were then exposed to the GlcNAc block and continued to completion as described in the Materials and Methods.
Supplemental Figure 4. Global O-GlcNAc and OGT levels are increased in PAECs. A Representative Western blot for the Global O-GlcNAc and OGT examined in multiple patient PAECs, control and IPAH (n=3). As a loading control, β-Actin was used.
Supplemental Figure 5. Chemical inhibition of OGT by Alloxan reduces cell proliferation in PAH PASMCs. Control and IPAH PASMCs were plated and treated with and without alloxan monohydrate (5mM) for 24hrs, followed by BrdU incorporation for 2 hours. The cells were subsequently collected and subjected to flow cytometry analysis (see Supplemental Materials and Methods).
Supplemental Figure 6. A longer exposure of the Immunoblot for HCF-1 as denoted in Figure 4. (A) siRNA specific KD of OGT and (B) an inhibitor to OGT under the conditions specified.
Supplemental Figure 7. Elevated OGT levels leads to an increase in HCF-1 cleavage/activation in IPAH that is analogous to the cancer cell mechanism. A549 and HeLa cells were treated in the presence (+) or absence (-) of OGT inhibitor (TT40) for 24 hours and HCF-1 was analyzed. Arrowheads represent the precursor HCF-1 and open arrows indicate HCF-1 specific cleavage products generated by OGT. A non-specific, but antibody reactive band is not marked in figure.
REFERENCES:

