Pharmacologic and genetic inhibition of G6PD activity attenuates right ventricle pressure and hypertrophy elicited by hypoxia and VEGF inhibitor+hypoxia

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Abstract

Background and Purpose: Pulmonary hypertension (PH) is a disease of hyperplasia of pulmonary vascular cells. The pentose phosphate pathway (PPP) – a fundamental glucose metabolism pathway – is vital for cell growth. Because treatment for PH is inadequate, our goal was to determine whether inhibition of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the PPP, prevents maladaptive gene expression that promotes smooth muscle cell (SMC) growth, reduces pulmonary artery remodeling, and normalizes hemodynamics in experimental models of PH. Experimental Approach: PH was induced in mice by exposure to 10% oxygen (Hx) or weekly injection of vascular endothelial growth factor receptor blocker (Sugen5416 (SU); 20 mg.kg-1) during exposure to hypoxia (Hx+SU), and in rats by injecting SU, exposure to 3 weeks Hx, and then 5 weeks normoxia. G6PD inhibitor (PDD4091;1.5 mg kg-1) was injected daily during exposure to Hx. We measured right ventricular (RV) pressure and left ventricular (LV) pressure-volume relationships, and gene expression in lungs of normoxic, Hx, and Hx+SU, and G6PD inhibitor-treated, mice and rats. Key Results: RV systolic and end-diastolic pressures were higher in Hx and Hx+SU than normoxic-control mice. Hx and Hx+SU decreased expression of epigenetic modifiers, increased hypomethylation of the DNA, and induced aberrant gene expression in lungs. G6PD inhibition decreased maladaptive expression of genes and SMC growth, reduced pulmonary vascular remodeling, and decreased RV pressures, compared to untreated PH groups. Conclusions and Implications: Inhibition of G6PD efficaciously reduces RV pressure overload in Hx and Hx+SU mice and rats and appears to be a safe pharmacotherapeutic strategy.

Introduction

Pulmonary hypertension (PH) is a multifactorial disease that is defined as sustained elevation of pulmonary arterial pressure (Farber & Loscalzo, 2004). The elevation of pulmonary arterial pressure increases right ventricular (RV) afterload, leading to heart failure and death (Runo & Loyd, 2003). The main vascular changes in PH are vasoconstriction, vascular cell proliferation, and thrombosis. Based on these findings, current standard of care is treatment with vasodilators. However, vasodilators such as endothelin receptor blockers, nitric oxide/nitrates, prostacyclin, and phosphodiesterase-5 inhibitors, fail to reverse vascular remodeling, and the long-term prognosis remains poor (Lajoie et al., 2016).

The pathogenesis of PH is still unclear. PH occurs under sustained environmental stress such as inflammation, shear stress, and hypoxia. This stress-stimuli contributes to the shifting of pulmonary vascular cells to hyperproliferative and apoptotic-resistant phenotypes allowing abnormal vascular remodeling and PH development (Boucherat, Vitry, Trinh, Paulin, Provencher & Bonnet, 2017; D'Alessandro et al., 2018). Pulmonary

vascular cells in patients with PH also undergo metabolic adaptation to support their high rate of proliferation or inadequate rates of mitotic fission. This metabolic shift, the Warburg phenomenon (Warburg, Wind & Negelein, 1927), is a failure of mitochondrial respiration and activation of aerobic glycolysis.

The pentose phosphate pathway (PPP) – a branch of glycolysis and a fundamental glucose metabolism pathway – is vital for cell growth and survival. Glucose-6-phosphate dehydrogenase (G6PD) is the first and rate-limiting enzyme of the PPP. G6PD and the PPP generate pentose sugar, which is required for the *de novo* cellular synthesis of RNA and DNA, and NADPH, a key cofactor for reductive and anabolic reactions (Gupte & Wolin, 2012). Recently, we found that inhibition and knockdown of G6PD in lungs of a chronic hypoxia-induced PH mouse model reduced and reversed: 1) Warburg phenomenon, 2) epigenetic modification (DNA methylation), 3) maladaptive expression of genes that support pulmonary artery remodeling, and 4) PH and left heart dysfunction (Joshi et al., 2020). However, the role of G6PD in the pathogenesis of hypoxia+Sugen5416–induced PH is unknown. Furthermore, whether inhibition of G6PD reduces remodeling of pulmonary artery and PH in hypoxia+Sugen5416 mouse model remains to be determined. We hypothesized that G6PD is a safe pharmacotherapeutic target to reduce PH in hypoxia+Sugen5416 mouse model. Therefore, our objectives were to determine whether the inhibition of G6PD activity by pharmacologic or genetic manipulations would decrease differential DNA methylation and maladaptive gene expression in lungs and pulmonary vascular cells, reduce vascular remodeling, and normalize hemodynamics in models of chronic hypoxia- and hypoxia+Sugen5416-induced PH.

Materials and methods

Drugs and reagents: All chemicals and reagents were purchased from Sigma, Thermo-Fisher, and VWR.

Animal models and experimental protocols: All animal experiments were approved by the New York Medical College Animal Care and Use Committee. Male and female C57BL/6J mice (18-32 g) were purchased from the Jackson Laboratory and randomly divided into four groups: normoxia (Nx), normoxia+Sugen5416 (Nx+SU), hypoxia (Hx), and hypoxia+Sugen5416 (Hx+SU) groups. Mice in Nx group were placed in normoxic (21% O_2) environment. Hx group was placed in a normobaric hypoxic chamber (10% O_2) for 6 weeks. Mice in Hx+SU group received subcutaneous injection of SU5416 (20 mg/kg) once weekly during 3 weeks of Hx (10% O_2). We also separated each group into non-treatment (Nx, Hx, and Hx+SU) and drug treatment (Nx+4091, Hx+4091 and Hx+SU+4091) groups. Mice in the drug treatment groups received daily subcutaneous injection of a novel G6PD inhibitor, N-[(3β,5α)-17-Oxoandrostan-3-yl]sulfamide (PDD4091; 1.5 mg kg^{-1} day^{-1}) (Hamilton et al., 2012), for 3 weeks. To determine whether PDD4091 reduces PH in a dose-dependent manner, mice were randomized to receive low (0.15 mg kg^{-1} day^{-1}), medium (1.5 mg kg^{-1} day^{-1}), or high (15 mg kg^{-1} day^{-1}) dose injection of PDD4091. In addition, we also determined the role of G6PD in the pathogenesis of SU+Hx+Nx-induced PH in rats. Wild-type (Sprague Daley; SD; 460-800 gm) rats and rats expressing loss-of-function Mediterranean *G6pd* variant ($G6PD^{S188F}$; 350-750 gm), which were recently generated in our laboratory (Kitagawa et al., 2020; in press), received one subcutaneous injection of SU (20 mg/kg) and then exposed to 3 weeks of Hx (10% O_2) followed by 5 weeks of Nx (SU/Hx/Nx). Hx and Hx+SU mice and rats are pre-clinical models of PH (Stenmark, Meyrick, Galie, Mooi & McMurtry, 2009). At the end of the treatment period, hemodynamic measurements were performed, tissue (lungs, hearts, and arteries) were harvested, and blood samples were collected. Data analysis was performed in blinded fashion.

Hemodynamic measurements: Closed-chest cardiac catheterization was performed using an MPVS Ultra Single Segment Pressure-Volume Unit (Millar Instruments, US) in combination with a cardiac catheter. Briefly, mice were anesthetized with isoflurane (induced at 3% and maintained at 1.5%) and placed on a heated table. RV systolic pressure (RVSP) and RV end-diastolic pressure (RVEDP) were measured by catheterization of the RV via the right external jugular vein using Millar Mikro-Tip catheter (Model SPR-671, tip size of 1.4F, Millar Instruments, US). The catheter was then removed, and the jugular vein was tied off. For hemodynamic measurements from LV, the right carotid artery was dissected and a Millar Mikro-Tip conductance catheter (model SPR-839, tip size of 1.4F, Millar Instruments, US) introduced into the artery and advanced into the LV *via* the aortic valve. Once steady-state hemodynamics were achieved, pressure-volume (P-V) loops were recorded and analyzed using LabChart 8 software (ADInstruments, US).

Hematocrit measurements and blood chemistry analysis: After hemodynamic measurements were completed, blood was collected from the cardiac chambers into a heparinized syringe. Heparinized blood was placed in capillary tubes, and hematocrit (%) was calculated as the length of the erythrocyte layer divided by the length of the entire blood sample. Plasma was shipped to Antech Diagnostics (NC, USA), a GLP facility, where blood tests were performed with routinely used clinical laboratory diagnostic tools.

Assessment of right ventricular hypertrophy: Following the cardiac catheterization, the animals were euthanized by cervical dislocation and whole hearts were excised and RV free wall and LV including ventricular septum (S) were separated and weighed independently. Fulton's index (RV/LV + S ratio) was calculated as an index of RV hypertrophy.

Isolation of small intrapulmonary arteries (IPA) and IPA tone measurements: Mice (25-30g) were sacrificed by cervical dislocation and small intrapulmonary arteries (IPA) of 3rd order (100–150 μm in diameter) were isolated from the lung, dissected free of connective tissue, and placed in Krebs bicarbonate buffer solution (pH 7.4) containing the following (in mM): 118 NaCl, 4.7 KCl, 1.5 $CaCl_2$ x$2H_2O$, 25 $NaHCO_3$, 1.1 $MgSO_4$, 1.2 KH_2PO_4, 5.6 glucose, and 10 HEPES. Then the vessels were mounted on a wire myograph (Danish Myo Technology A/S, Aarhus, Denmark) and bathed in Krebs buffer solution at 37°C and an optimal passive tension of 3 mN. After 30 min of incubation, the arterial viability and equilibration were assessed by the stimulation of the vessels with repeated 10 min exposures to KCl (60 mM; 60K). For registration of vascular ring contractile activity and its following analysis, Chart 5.5.4 and LabChart Reader 8.1.9 (ADInstruments, Inc.) software were used. Vascular tension is presented as a percentage of the maximum steady-state contraction level obtained to the exposure to 60K.

Quantitative real-time PCR: Real time RT-PCR technique was used to analyze mRNA expression. Briefly, total RNA was extracted from lungs using a Qiagen miRNEasy kit (Cat # 217004). The input RNA quality and concentration were measured on the Synergy HT Take3 Microplate Reader (BioTek, Winooski, VT) and cDNA was prepared using SuperScript IV. VILO Master Mix (Cat # 11756500, Invitrogen) for mRNA. Quantitative PCR was performed in duplicate using TaqManTM Fast Advanced Master Mix (Cat # 44-445-57) for mRNA using a Mx3000p Real-Time PCR System (Stratagene, Santa Clara, CA). The primers for the QPCR were purchased from Thermo Fisher Scientific/TaqMan. Results for mRNA expression was normalized to internal control *Tuba1a* , and relative mRNA expression was determined using the ΔCt method.

RNA-Seq analysis: After collecting lungs from pulmonary normotensive and hypertensive mice, total RNA was isolated from tissue using the Qiagen All Prep DNA/RNA/miRNA Universal kit according to manufacturer's instructions. RNA was quantified using the NanoDrop (ThermoFisher) and quality was assessed using the Agilent Bioanalyzer 2100. RNA-seq library construction was performed using the TruSeq Stranded Total RNA Preparation kit (Illumina) with 200 ng of RNA as input according to the manufacturer's instructions. Libraries were sequenced on the HiSeq2500 with single-end reads of 100nt at the University of Rochester Genomics Research Center. Single-end sequencing was done at a depth of 10 million reads per replicate (N=3). Quantitative analysis, including statistical analysis of differentially expressed genes, was done with Cufflinks 2.0.2 and Cuffdiff2 (http://cufflinks.cbcb.umd.edu). The Benjamini-Hochberg method was applied for multiple test correction (FDR<0.05).

Reduced Representation Bisulfite Sequencing (RRBS): Genomic DNA was isolated from lungs using the Qiagen All Prep DNA/RNA/miRNA Universal kit according to manufacturer's instructions. DNA was quantified using the NanoDrop (ThermoFisher) and Qubit Fluorometer (ThermoFisher). Genomic DNA quality was assessed using the Agilent TapeStation. RRBS library construction was performed with the Premium RRBS Kit (Diagenode) following the manufacturer's instructions. Libraries were sequenced on the HiSeq2500 with paired-end reads of 125nt. Raw reads generated from the Illumina HiSeq2500 sequencer were demultiplexed using bcl2fastq version 2.19.0. Quality filtering and adapter removal are performed using Trim Galore version 0.4.4_dev with the following parameters: " –paired –clip_R1 3 – clip_R2 3 –three_prime_clip_R1 2 –three_prime_clip_R2 2" (http://www.bioinformatics.babraham.ac.uk/projects/ trim_galore/). Processed and cleaned reads were then mapped to the mouse reference genome (mg38) using Bismark version 0.19.0 with the following parameters: " –bowtie2 –maxins 1000".11 Differential methylation analysis was performed

using methylKit version 1.4.0 within an R version 3.4.1 environment.12 Bismark alignments were processed via methylKit in the CpG context with a minimum quality threshold of 10. Coverage was normalized after filtering for loci with a coverage of at least 5 reads and no more than the 99.9th percentile of coverage values. The coverage was then normalized across samples and the methylation counts were aggregated for 500nt windows spanning the entire genome. A unified window set across samples was derived such that only windows with coverage by at least one sample per group were retained. Differential methylation analysis between conditional groups was performed using the Chi-squared test and applying a qvalue (SLIM) threshold of 0.05 and a methylation difference threshold of 25 percent.

Cell Culture: Human pulmonary artery smooth muscle cell (PASMCs; Purchased from Lonza, USA) were maintained at 37°C under 5% CO_2 in smooth muscle basal media (Lonza, #CC-3181) supplemented with growth factors (SMGM-2 smooth muscle singlequots kit, Lonza, #CC-4149). Once cells reached approximately 70% confluence, they were sub-cultured using 0.05% trypsin-EDTA (GIBCO, Cat # 25300-054, Thermo Fischer Scientific, Grand Island, NY) into 6-well plates at about $3x10^5$ cells/well.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 5 software. Values are presented as mean ± standard error (SE). Statistical comparisons of samples were performed with Student's t test for comparing two groups. Multiple comparisons were performed by one-way ANOVA followed by Sidak's post hoc test. Difference with $P<0.05$ between the groups was considered significant.

Results

G6PD inhibition decreased chronic Hx- and Hx+SU-induced PH in mice: PH was induced by exposing C57BL/6J mice to Hx and Hx+SU (Figure 1A). C57BL/6J mice exposed to Hx and Hx+SU had higher RVSP, RVEDP, and arterial elastance (Ea) than Nx mice (Figure 1B and Table 1). In Hx+SU group, RVSP, RVEDP, Ea, and LV stiffness were higher than those of Hx group (Figure 1C and D, and Table 1). Furthermore, cardiac index (CI) was decreased in Hx and Hx+SU, and more so in Hx+SU mice (Table 1).

In Hx mice, we first established the maximum tolerated dose (MTD) of G6PD inhibitor (4091). The MTD in Hx mice was 15 $mg.kg^{-1}.day^{-1}$ beyond which PDD4091 depressed LV function. More importantly, PDD4091 had a reasonably wide therapeutic window (0.01 to 15 $mg.kg^{-1}.day^{-1}$) with an EC_{50} of 0.26±0.10 and 0.58±0.36 $mg.kg^{-1}.day^{-1}$ reduced both RVSP and RVEDP. G6PD inhibitor, PDD4091, treatment to Hx mice decreased the elevated RVSP and RVEDP in a dose-dependent manner (Figure 2C and D top panel). Moreover, PDD4091 (1.5 $mg.kg^{-1}.day^{-1}$) treatment to both Hx and Hx+SU mice efficaciously and comparably reduced (by 50%) the elevated RVSP and RVEDP (Figure 2C and D bottom panel) and Ea (Table 1). Fulton's index was increased in Hx and Hx+SU groups compared with Nx group. G6PD inhibitor reduced elevated Fulton's index in Hx and Hx+SU groups (Table 1), and increased CI in both groups (Table 1).

G6PD inhibitor did not cause toxicity in mice: To examine whether G6PD inhibitor caused toxicity in mice, we measured hematocrit and performed blood chemistry analysis to assess organ (such as; liver, pancreas, and kidney) damage/function. As expected, mice exposed to Hx and Hx+SU had higher hematocrit than their respective controls, Nx and Nx+SU (Table 1). Treating Hx and Hx+SU mice with PDD4091 (1.5 $mg.kg^{-1}.day^{-1}$) for 3 weeks reduced the elevated hematocrit to the Nx levels in Hx, but not in Hx+SU, mice (Table 1). The blood chemistry revealed that PDD4091 treatment normalized electrolyte levels and did not cause toxicity in mice (Table 2).

Loss-of-function Mediterranean *G6pd* variant normalized RV pressures and decreased RV hypertrophy elevated by SU/Hx/Nx exposure in rats: Although Hx+SU mouse model is used to study pathogenesis of PH, it is not a perfect experimental model of human PH (Vitali et al., 2014). Therefore, we also tested our hypothesis in a SU/Hx/Nx rat model. PH was induced by exposing rats to SU/Hx/Nx, as shown in figure 2A, in Wild-type SD rats and in the $G6PD^{S188F}$ rat model mimicking human condition (Kitagawa et al., 2020; in press). These $G6PD^{S188F}$ rats have less G6PD activity in the heart and lungs as compared to wild-type SD rats (Figure 2B). SU/Hx/Nx increased RVSP (by 69.8% ; Figure 2C) and Fulton's index (by 32.2%; Figure 2E), compared to Nx wild-type SD rats. Intriguingly, RVSP (Figure 2C), RVDP (Figure 2D), and RV hypertrophy (Figure 2E), were reduced significantly in SU/Hx/Nx $G6PD^{S188F}$ rats as

compared to SU/Hx/Nx wild-type SD rats.

G6PD inhibition relaxed pre-contracted PA, decreased PASMC growth, and reduced PA remodeling in Hx+SU mice: PA remodeling is the hallmark of severe PH. Hyperplasic and apoptosis-resistant PA endothelial cells and PASMCs contribute to hypertensive remodeling (Morrell et al., 2009). Previously, we and others proposed that SMCs switch from a differentiated to a dedifferentiated phenotype in PA of hypertensive patients and animals and contribute to PA remodeling (Chettimada, Gupte, Rawat, Gebb, McMurtry & Gupte, 2015; Sahoo et al., 2016; Zhou, Negash, Liu & Raj, 2009). Dedifferentiated SMCs are hyper-proliferative, migratory, and secretory (Frismantiene, Philippova, Erne & Resink, 2018). Previous studies show that the Hx+SU mouse model of PH has more severe PA remodeling than Hx mice (Vitali et al., 2014). Therefore, we determined whether G6PD inhibition relaxes PA in *ex vivo* studies, stunts the growth of PASMCs exposed to Hx and SU in cell culture, and reduces remodeling of PA in Hx+SU mice. Our results demonstrated PDD4091 dose-dependently relaxed PA pre-contracted with KCl (Figure 3A). Application of PDD4091 (1 μmol/L) for 48 hours to PASMCs cultured in normoxia decreased cell numbers (Figure 3B) and in addition attenuated the cell growth evoked by Hx and Hx+SU (Figure 3C). Treatment of Hx+SU mice with PDD4091 (1.5 $mg.kg^{-1}.day^{-1}$) for 3 weeks abrogated the occlusive pulmonary vascular remodeling (Figure 3D).

Gene expression is altered in lungs of Hx and Hx+SU mice: To discover the genetic and epigenetic determinants of PASMC growth in the PA wall and remodeling of PA in Hx and Hx+SU, we first performed RNA-seq analysis in lungs of mice exposed to Nx, Hx, and Hx+SU. The results revealed that out of 159 and 97 genes upregulated in lungs of Hx vs Nx and Hx+SU vs Nx mice, respectively, only 3 genes were commonly upregulated in both groups (Figure 4A). Whereas 1511 and 1523 genes were downregulated in lungs of Hx vs Nx and Hx+SU vs Nx mice, respectively, 1085 genes were commonly downregulated in both groups (Figure 4A). Transcription factor binding site enrichment analysis using oPOSSUM (Kwon, Arenillas, Worsley Hunt & Wasserman, 2012) disclosed TCFCP2l1, KLF4, and E2F1 as the most enriched TFBS in genes upregulated in the Hx group and HIF1A::ARNT, KLF4, and SP1 as the most enriched TFBS in genes upregulated in the Hx+SU group (Figure 4B top panels). HOXA5, PDX1, and PRRX2 were the most enriched TFBS in genes downregulated in the Hx and Hx+SU groups (Figure 2B bottom panels). Suppressor of fused (*Sufu*) homolog and Cytochrome P450 1A1 (*Cyp1a1*) genes were, respectively, upregulated >100- and >15-fold in lungs of mice exposed to Hx+SU, but not to Hx, while all genes downregulated >20-fold were common in lungs of mice exposed to Hx+SU and Hx (Figure 4C).

Methylation of DNA is decreased in lungs of Hx and Hx+SU mice: Epigenetic modifications, including methylation of DNA, are incriminated in the pathogenesis of PH (Cheng, Wang & Du, 2019; Joshi et al., 2020). Recently, we reported that downregulation of ten-eleven translocation 2 (*Tet2*) DNA demethylase in lungs of Hx Sv129J mice lacking *Cyp2c44* gene contributes to the genesis of PH, and also demonstrated that inhibition of G6PD was ineffective in reducing PH in hypoxic $Tet2^{-/-}$ mice (Joshi et al., 2020). Therefore, we postulated that downregulation of *Tet2* by Hx may augment DNA methylation in C57BL/J mice and mediate maladaptive gene expression. Unexpectedly, we found that expression of *Tet1* but not of *Tet2* or *Tet3* was reduced in lungs of C57BL/J mice exposed to Hx (Figure 5A and Table 3). In addition, expression of*Dnmt3b* but not of *Dnmt3a* or *Dnmt1a* was decreased (Figure 5A and Table 3). Concomitantly, we found that 45,321 CpG regions genome-wide were differentially methylated, and out of which 46.53% regions were hypermethylated and 53.47% were hypomethylated in lungs of mice exposed to Hx as compared to Nx (Figure 5B). While 46,286 CpG regions genome-wide were differentially methylated, and out of which 46.15% regions were hypermethylated and 53.85% were hypomethylated in lungs of mice exposed to Hx+SU as compared to Nx (Figure 5C). Therefore, there were 0.38% more hypomethylated and less hypermethylated CpG regions in lungs of C57BL/J mice exposed to Hx+SU than Hx. Furthermore, two genes, *Cyp1a1* and *Kcng3* , out of 12 differentially regulated genes by Hx and Hx+SU (Figure 4C), were hypomethylated in lungs of both Hx and Hx+SU mice (Figure 5D). It is noteworthy that CpG regions 2890 bp from the transcription start site of *Cyp1a1* gene were hypomethylated by Hx and Hx+SU (Table 4).

G6PD inhibition decreased expression of *Cyp1a1* and*Sufu* genes in lungs of mice and in hu-

man PASMCs exposed to Hx+SU : Next, we determined whether inhibition of G6PD activity, which hypermethylated *Cyp1a1* gene in lungs of Hx mice (Table 4), decreased expression of *Cyp1a1* and *Sufu* in lungs of Hx and Hx+SU mice and in human PASMCs exposed to Hx+SU. Treatment of PDD4091 (1.5 $mg.kg^{-1}.day^{-1}$) for 3 weeks to Hx mice and application of PDD4091 (1 μM) to human PASMCs for 48 hr rescinded the Hx+SU-induced *Cyp1a1* and *Sufu* expression in lungs (Figure 6A, B) and in human PASMCs (Figure 6C).

Discussion

The results of this study provided evidence that downregulation of the epigenetic modifiers *Tet1* and *Dnmt3b* and hypomethylation of DNA altered gene expression in lungs of Hx- and Hx+SU-induced PH mice. Furthermore, pharmacologic inhibition of G6PD activity relaxed pre-contracted PA, decreased growth of PASMCs evoked by Hx and SU, reduced expression of *Cyp1a1* and *Sufu* , which potentially arrested growth of PASMCs, and rescinded occlusion of PA in lungs of mice exposed to Hx+SU. Additionally, we demonstrated attenuation of SU/Hx/Nx-induced PH in a loss-of-function Mediterranean *G6pd*variant rat model. These results suggest G6PD is a common factor for the genesis of PH in Hx and Hx+SU mice and rats. Since a selective inhibitor of G6PD activity decreased occlusive remodeling of PA and alleviated PH induced by Hx and Hx+SU in mice without causing toxicity, we propose that G6PD might be a safe pharmacotherapeutic target to reduce PH in humans.

Hx and Hx+SU rat and mouse models are routinely used to study the pathology of PH (Stenmark, Meyrick, Galie, Mooi & McMurtry, 2009). We observed in this study that mice exposed to Hx for 6 weeks and to Hx+SU for 3 weeks developed PH, which was more severe in Hx+SU than Hx group. In chronically Hx (3 weeks) mice, vasoconstriction and muscularization of small arteries, but not obliterative remodeling of PA, contribute to increased pulmonary arterial pressure and RV pressure overload (Stenmark, Meyrick, Galie, Mooi & McMurtry, 2009). The more severe PH in Hx+SU mice is attributed to the formation of angio-obliterative lesions in addition to vasoconstriction and muscularization (Vitali et al., 2014). To support our findings of pharmacologic G6PD inhibition in the Hx+SU mice, we also used the SU/Hx/Nx rat model of PH and found that the hypertension was reduced in rats expressing $G6PD^{S188F}$, a Mediterranean *G6pd* variant that has 80% less activity than wild-type G6PD. Thus, our results indicate that inhibition of G6PD activity by either pharmacologic or genetic interventions reduces remodeling of PA and elevated RV pressure/overload in PH mice and rats.

The above observations raise the question of whether the underlying genetic determinants of PH in mice exposed to Hx and Hx+SU are same or different? To seek answers, we performed RNA-seq analysis in lungs which revealed that >1000 downregulated genes and only 3 upregulated genes, driven by different transcription factors, were common between the two models. Most striking difference was noticed in >15-fold increase of *Sufu* and *Cyp1a1* genes in lungs of mice exposed to Hx+SU but not to Hx. Furthermore, exposure to SU increased expression of both *SUFU* and *CYP1A1* genes in Hx but not in Nx human PASMCs. While these results are consistent with a recent study that indicates HIF::ART-driven *Cyp1a1* gene is upregulated in lungs of rats exposed to SU/Hx/Nx and in human PASMCs by SU (Dean et al., 2018), an increase of *Sufu* in lungs of PH mice and human PASMCs has not been reported. CYP1A1 is an estrogen-metabolizing enzyme that produces mitogenic metabolites of estrogen in human PASMCs (Dean et al., 2018) and SUFU is a negative regulator of hedgehog signaling, which controls cell proliferation during development in invertebrates and vertebrates (Briscoe & Therond, 2013; Liu, 2019). Increased CYP1A1 contributes to the pathogenesis of PH in SU/Hx/Nx rats (Dean et al., 2018). Our results suggest that increased CYP1A1 and SUFU signaling may have a potential role in the genesis of occlusive lesion formation in Hx+SU mice. Since transcription of*CYP1A1* was arrested and that of *SUFU* was partially decreased in mice lungs and in human PASMCs by G6PD inhibition, transcription of *CYP1A1* and *SUFU* genes in lungs and PASMCs exposed to Hx+SU is potentially controlled by G6PD. Therefore, we propose inhibition of G6PD activity could be useful in reversing the elevated expression of the pathogenic *CYP1A1* and *SUFU*genes in PH.

We and others have recently proposed that DNA methylation and other epigenetic modifications potentially promote aberrant/maladaptive gene expression, a determinant of inflammatory and hyperproliferative cell

phenotype, in remodeled PA (Hu, Zhang, Laux, Pullamsetti & Stenmark, 2019; Joshi et al., 2020). Furthermore, we recently showed that expression of *Tet2* , a DNA demethylase considered as a master regulator of differentiated fate of SMC phenotype (Liu et al., 2013), was downregulated in lungs of Sv129J mice with a *Cyp2c44* gene knockout (Joshi et al., 2020). Therefore, we assumed that a loss of TET2 modifies DNA methylation and initiates maladaptive gene expression in lungs of mice exposed to Hx and Hx+SU. Unexpectedly, expression of*Tet1* , but not of *Tet2,* and *Dnmt3b* was downregulated in lungs of C57BL/J mice exposed to Hx and Hx+SU. We propose genetic variations and differences in gene regulation between Sv129J and C57BL/J mice (Hashimoto et al., 2020) may be the cause of *Tet1* and*Dnmt3b* downregulation, but not of other isoforms of DNA demethylases and methyltransferases, in response to stress observed in C57BL/J mice. Since G6PD inhibition prevented downregulation of*Tet1* and *Dnmt3b* in lungs of Hx mice, it appears that G6PD, directly or indirectly, suppressed transcription of *Tet1* and*Dnmt3b* in lungs of Hx and Hx+SU C57BL/J mice. TET proteins are involved in the regulation of hematopoietic stem cell homeostasis, and hematological malignancies and diseases (Nakajima & Kunimoto, 2014). Although loss of single TET protein is not sufficient to promote malignancies (An et al., 2015), TET1 and TET2 have been shown to, respectively, repress and promote osteogenesis and adipogenesis (Cakouros et al., 2019). Furthermore, inhibition of TET1 blocks expression of large-conductance Ca^{2+}-activated K^+ channel β1 subunit in uterine arteries of pregnant rats (Hu et al., 2017). Expression of this channel is a marker of differentiated SMCs. Therefore, downregulation of *Tet1* could imply that: 1) SMCs are dedifferentiated and 2) decreased Ca^{2+}-activated K^+ channels contribute to constrict PAs and increase pressure in lungs of Hx and Hx+SU mice. Therefore, altogether these results suggest that DNA methylation modulated by G6PD is functionally important in gene regulation and substantiate our previous finding that G6PD is a regulator of DNA methyltransferases and demethylase, which plays a crucial role in remodeling of PA (Joshi et al., 2020).

Transcription of the many genes, including the *Cyp1a1* gene that promotes PASMC proliferation (Dean et al., 2018), was repressed through hypermethylation of the DNA evoked by G6PD inhibition. In contrast, transcription of *Sufu* in mouse lungs evoked by Hx+SU was not regulated by the methylation of DNA. These results suggest G6PD inhibition activated other mechanisms of gene expression in addition to differential methylation of the DNA, and these mechanisms worked independently but synergistically to regulate gene expression in lungs of Hx and Hx+SU mice.

In addition to arresting maladaptive gene expression in vascular cells of the PA wall and reducing cell growth in occlusive pulmonary arterial disease, G6PD inhibitor, PDD4091, dose-dependently relaxed pre-contracted PAs. By using 17-ketosteroids (dehydroepiandrosterone (DHEA) and epiandrosterone – a DHEA metabolite), which inhibit G6PD activity, and siRNA-mediated knockdown of *G6pd* , we have previously shown that reduced G6PD elicits relaxation of pre-contracted pulmonary artery (Gupte, Li, Okada, Sato & Oka, 2002) and reduces RV pressures in Hx and SU/Hx/Nx rats (Chettimada, Gupte, Rawat, Gebb, McMurtry & Gupte, 2015; Chettimada et al., 2012). Recently, we found that arteries of $G6PD^{S188F}$ rats as compared to wild-type SD rats constrict less in response to nitric oxide synthase inhibitor and L-type Ca^{2+} channel opener (Kitagawa, 2020). Therefore, these studies and our current findings collectively suggest that G6PD inhibition reduces the elevated RV pressures in Hx- and Hx+SU-induced PH by dilating PAs and reducing PA remodeling.

In conclusion, our results collectively demonstrate that G6PD activity is an important contributor to differential DNA methylation, aberrant/maladaptive gene expression, and remodeling of PA in Hx and Hx+SU mice. The inhibition of G6PD activity abrogated pulmonary vascular cell remodeling *in vivo* . As a consequence, the inhibition of G6PD activity by pharmacologic and genetic manipulations improved the hemodynamics in mouse and rat models of PH. Therefore, G6PD inhibitor, N-[(3β,5α)-17-Oxoandrostan-3-yl]sulfamide (PDD4091), might be employed in the future as a pharmacotherapeutic agent to treat different forms of PH.

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Table 1: Hemodynamics changes in normoxia±PDD4091, hypoxia±PDD4091, hypoxia+SU5149±PDD4091 mice.

	Nx (n = 11)	**Nx+4091 (n = 6)**	**Hx (n = 9)**	**Hx+4091 (n = 11)**	**Hx+SU (n**
BW (g)	23 ± 3	26 ± 5	24 ± 3	23 ± 3	25 ± 3
HR (bpm)	455 ± 71	508 ± 71	455 ± 36	466 ± 34	519 ± 19
LVSP (mmHg)	104 ± 11	96 ± 4	105 ± 6	97 ± 4	102 ± 21
LVEDP (mmHg)	11 ± 3	6 ± 3	15 ± 3	11 ± 2	17 ± 10
LVESV (uL)	40 ± 9	46 ± 11	35± 8	37± 11	42 ± 15
LVEDV (uL)	55 ± 10	68± 10*	50 ± 9	58 ± 12	51 ± 13
CI (mL/m2/min)	1092 ± 270	1495±142**	850 ± 138*	1413 ± 303§§§	703 ± 76**
Ea (mmHg/uL)	5.6 ± 1.0	3.8 ± 0.4***	7.2 ± 1.0*	4.3 ± 0.9§§§	9.2 ± 1.5**
dP/dt$_{max}$ (mmHg/s)	5841 ± 803	6385 ± 462	5559 ± 496	5567 ± 668	6021 ± 111
-dP/dt$_{min}$ (mmHg/s)	5266±1029	5890 ± 570	4945 ± 788	4753 ± 553	4648 ± 109
dP/dV$_{max}$ (mmHg/uL)	8.5 ± 2.8	6.5 ± 1.6	8.7 ± 2.8	6.1 ± 2.2§	15.7 ± 4.2*
Tau (ms)	12 ± 4	8 ± 2*	13 ± 4	11 ± 3	12 ± 2
Fulton's index, RV/LV+S	0.23 ± 0.04	0.25 ± 0.04	0.39±0.08***	0.20±0.03§§§	0.39±0.03**
Ht (%)	46 ± 3	44 ± 2	60 ± 3***	48 ± 6§§§	59 ± 3***

Table 2: Blood chemistry in mice.

Blood Parameters	Nx	Hx	Hx+PDD4091 (1.5 $mg.Kg^{-1}.day^{-1}$)
	N=3	N=5	N=3
Blood urea nitrogen (mg/dl)	31.3±3.8	31.8±1.4	36.0±2.0
Creatinine (mg/dl)	0.23±0.03	0.20±0.0	0.23±0.03
Glucose (mg/dl)	115±33	127±18	145±19
Na^+ (mmol/L)	162±1	182±2*	168±3#
K^+ (mmol/L)	4.6±0.4	3.5±0.1*	4.8±0.4#
Cl^- (mmol/L)	125±1	118±1*	127±3#
Alkaline phosphatase (U/L)	49±4	68±8*	40±4#
Alanine aminotransferase (U/L)	9±2	6±1	5±2
Aspartate aminotransferase (U/L)	75±6	106±28	75±2
Total bilirubin (mg/dl)	0.1±0.0	0.1±0.0	0.1±0.0
Direct bilirubin (mg/dl)	0±0	0±0	0±0
Lactate dehydrogenase (U/L)	374±78	367±39	449±22
Creatine kinase (U/L)	254±57	268±68	136±21
Gama gutamyltransferase (U/L)	0±0	0±0	0±0
Total protein (g/dl)	3.6±0.1	3.2±0.1*	3.7±0.1#
Albumin (g/dl)	2.1±0.1	1.1±0.1*	2.1±0.1#
Ca^{2+} (mg/dl)	7.6±0.2	6.6±0.1*	7.2±0.2#
PHOS (mg/dl)	9.2±1.7	8.3±0.9	9.4±1.2
Mg^+ (mg/dl)	2.2±0.1	2.2±0.1	2.3±0.3
Cholesterol (mg/dl)	44±8	52±3	66±7
Triglycerides (mg/dl)	46±13	82±15	64±9
Amylase (U/L)	406±64	300±13	387±34
Lipase (U/L)	87±20	45±6*	42±7*

*P<0.05 vs Nx and #P<0.05 vs Hx

Table 3: Expression of *Dnmt* and *Tet* genes in mouse lungs.

Condition	Gene Name	Strand	Distance from TSS	% Differential Methylation
Hx vs Nx	ENSMUST00000034865.4_Cyp1a1	+	2890	-36.1
Hx+SU vs Nx	ENSMUST00000034865.4_Cyp1a1	+	2890	-41.9
Hx+4091 vs Hx	ENSMUST00000034865.4_Cyp1a1	+	2890	36.1
Hx vs Nx	ENSMUST00000051482.1_Kcng3	-	36396	-26.7
Hx+SU vs Nx	ENSMUST00000051482.1_Kcng3	-	34396	-29.1
Hx+4091 vs Hx	ENSMUST00000051482.1_Kcng3	-	36396	26.7

Table 4: Methylation status of *Cyp1a1* and *Kcng2*gene in mouse lungs .

Condition	Gene Name	Strand	Distance from TSS	% Differential Methylation
Hx vs Nx	ENSMUST00000034865.4_Cyp1a1	+	2890	-36.1
Hx+SU vs Nx	ENSMUST00000034865.4_Cyp1a1	+	2890	-41.9
Hx+4091 vs Hx	ENSMUST00000034865.4_Cyp1a1	+	2890	36.1
Hx vs Nx	ENSMUST00000051482.1_Kcng3	-	36396	-26.7
Hx+SU vs Nx	ENSMUST00000051482.1_Kcng3	-	34396	-29.1
Hx+4091 vs Hx	ENSMUST00000051482.1_Kcng3	-	36396	26.7

Figure Legends:

Figure 1: Daily injection of a novel G6PD inhibitor, PDD4091, decreased right ventricle pressure, hypertrophy, and pulmonary artery remodeling in mice elicited by hypoxia and hypoxia+Sugen5416. A) A schematic showing various treatment protocols in C57BL/6 mice. B) Original tracing showing right ventricle pressure in mice treated with and without PDD4091 (1.5 $mg.kg^{-1}.day^{-1}$) for 3 weeks in normoxia and hypoxia, and mice treated with Sugen5416 (20 $mg.kg^{-1}$) once a week. Right ventricle systolic (RVSP; C) and diastolic (RVDP; D) were reduced by PDD4091 treatment for 3 weeks to hypoxic mice. N=6-11 in each group (male=3-6 and female=1-5). Statistical analysis was performed using Two-way ANOVA and Sidak's test for multiple comparisons.

Figure 2: Right ventricle pressure and hypertrophy is less in $G6PD^{S188F}$ compared to wild-type rats exposed to Sugen/Hypoxia/Normoxia. A) A schematic showing protocol of exposing rats to Sugen5416 (20 mg.kg-1) followed by hypoxia (10% O_2) for 3 weeks and normoxia (ambient air) for 5 weeks. B, C) Right ventricle systolic (RVSP) and diastolic (RVDP) pressure increased more in wild-type rats as compared to $G6PD^{S188F}$ rats exposed to Su/Hx/Nx than normoxic control. D) Right ventricle hypertrophy indicated as RV-to-LV+S ratio (Fulton's Index) increased in wild-type compared to $G6PD^{S188F}$ rats exposed to Su/Hx/Nx than normoxic control. N=4 in each group. All male rats were used in the experimental groups because *G6pd* is a X-linked gene. Statistical analysis was performed using Two-way ANOVA and Sidak's test for multiple comparisons.

Figure 3 : G6PD inhibitor, PDD4091, relaxed pre-contracted PA, decreased PASMC growth, and rescinded occlusive lesion in PA. A) Pulmonary arterial rings were contracted with KCl (30 mM) and application of PDD4091 relaxed the pre-contracted arterial rings in dose-dependent manner, (N=6 in each dose). B) Application of PDD4091 (1 μmol/L) to human pulmonary artery smooth muscle cells for 48 hours decreased growth of cells cultured in 21% O_2, N=6. C) Hypoxia (3% O_2; N=6) and Sugen (1 μmol/L; N=6) as compared to normoxia-control (21% O_2; N=6) increased growth of control human pulmonary artery smooth muscle cells, and application of PDD4091 (1 μmol/L) to cells in hypoxia (N=6) for 48 hr reduced cell growth. D) Immunofluorescent micrograph shows occluded pulmonary artery in lungs of mice exposed to hypoxia+Sugen5416 (SU), and occluded pulmonary arteries were not present in lungs of hypoxia+Sugen5416 (SU) mice treated with PDD4091 for 3 weeks. N=4 in normoxia; hypoxia+Sugen5416 (SU) and hypoxia+Sugen5416 (SU)+4091 groups. *P<0.05 vs $3x10^{-9}$ M in panel A. *P<0.05 vs control or normoxia (Nx), and #P<0.05 vs hypoxia in panel B and C. Statistical analysis was performed by One-way ANOVA in panel A and C, and by Student's t -test in panel B.

Figure 4 : Differential gene expression in lungs of mice exposed to hypoxia and hypoxia+Sugen. A) Venn diagram of whole-genome RNA-seq analysis demonstrate 3 genes are common in significantly up regulated cohort and 1085 genes are common in significantly down regulated cohort in lungs of mice exposed to hypoxia (Hx) and hypoxia+Sugen (Hx+SU) compared to normoxia-control (Nx). B) Transcription factor binding site enrichment analysis using oPPOSUM revealed TCFCP2L1 and KLF4 in Hx vs Nx and HIF1A::ARNT and KLF4 in Hx+SU vs Nx are the most enriched TFBS in genes up regulated category, and REST and HOXA5 in Hx vs Nx and HOXA5 and PDX1 in Hx+SU vs Nx are the enriched TFBS in genes down regulated category in mice lungs. C) Heat map of RNA-seq results demonstrate*Sufu* and *Cyp1a1* genes are most up regulated in lungs of mice exposed to Hx+SU vs Nx but not to Hx vs Nx, and *Tubg2* and*Sv2b* genes are most down regulated in lungs of mice exposed to Hx+SU vs Nx and to Hx vs Nx. N=3 in each group. RNA-seq

was performed on three lungs in each group. Male=2 and female=1. The Benjamini-Hochberg method was applied for multiple test correction (FDR<0.05).

Figure 5 . DNA methylation in lungs of mice exposed to hypoxia and hypoxia+Sugen5416. A) Expression of genes that encode ten-eleven translocation 1 demethylase (*Tet1*) and DNA methyltransferase 3b (*Dnmt3b*) is significantly decreased in lungs of mice exposed to hypoxia (Hx) and hypoxia+Sugen5416 (Hx+SU). Methylation of the DNA in lungs of mice exposed to normoxia, hypoxia and hypoxia+SU was determined by Reduced Representation Bisulfite Sequencing method. B, C) Pie graph of differentially methylated CpG regions demonstrate less regions are hyper- than hypo-methylated in lungs of mice exposed hypoxia (Hx) and hypoxia+Sugen (Hx+SU) vs normoxia-control (Nx). D) *Cyp1a1* and*Kcng3* genes were hypomethylated in lungs of Hx and Hx+SU mice. Differential methylation analysis between conditional groups was performed using the Chi-squared test and applying a qvalue (SLIM) threshold of 0.05 and a methylation difference threshold of 25 percent.

Figure 6 . Expression of *Cyp1a1* and *Sufu* genes is decreased by G6PD inhibitor, PDD4091. A, B) Real-time PCR results disclosed that *Cyp1a1* and *Sufu* expression are increased in lungs of mice exposed to Hx+SU but not to Hx, and PDD4091 treatment decreased *Cyp1a1* and *Sufu* . N=4, lungs from 3 different mice (male=2 and female=1) were used for RNA-seq analysis in each group, and N=5, lungs 5 different mice (male=3 and female=2) were used for qPCR analysis in each group. C) Expression of *CYP1A1* and *SUFU*increased in human pulmonary artery smooth muscle cells cultured in hypoxia (3 % O_2), but not in normoxia (21% O_2), by Sugen5416 (SU: 1 μmol/L). Application of PDD4091 (1 μmol/L) to cells for 48 hr rescinded their elevated expression of*CYP1A1* and *SUFU* . N=6 in each experimental condition. Statistical analysis was performed using Two-way ANOVA and Sidak's test for multiple comparisons.

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