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Is PKM2 Phosphorylation Prerequisite for Oligomer Disassembly in Pulmonary Arterial Hypertension?

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Short title: Novoyatleva – Pyruvate Kinase Muscle 2 (PKM2) in PAH

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Pulmonary arterial hypertension (PAH) is an incurable vascular disease characterized by pulmonary vascular remodeling and increased pulmonary vascular resistance, culminating in right ventricular failure(1). PAH shares major pathophysiological mechanisms with cancer, including substantial metabolic reprogramming with a shift towards glycolysis over mitochondrial oxidative phosphorylation (Warburg effect). The final step in glycolysis is catalyzed by the enzyme pyruvate kinase (PK). The PK muscle gene (*PKM*) is alternatively spliced to produce PKM1, which forms constitutively active tetramers in terminally differentiated tissues, and PKM2, which forms both high-activity tetramers and low-activity dimers/monomers in proliferating cells and tumors (2). Conformational changes of PKM2 that affect the dynamic equilibrium of monomers, dimers, and tetramers are tightly controlled by numerous allosteric effectors (e.g. fructose-1,6-bisphosphate) and posttranslational modifications (e.g. phosphorylation at Y105 and S37). A shift in the PKM2 equilibrium from tetramers to low-activity dimers/monomers ultimately diverts the glycolytic flux into biosynthetic routes and promotes Warburg effect. Thus, PKM2 oligomerization is a decision point that determines the ultimate activity of the enzyme, paving the way to metabolic reprogramming. Growth factor-stimulated ERK1/2-dependent phosphorylation leads to PKM2 nuclear translocation empowering the transcriptional activation of genes involved in metabolism (3). Furthermore, nuclear PKM2 acts as a coactivator of β-catenin to induce Cyclin D1 and c-Myc expression, highlighting the non-metabolic role of PKM2 for cell cycle progression (4). PKM2 nuclear function is regulated by poly-ADP ribose polymerase-1 (PARP-1) signaling pathway, established to play a crucial role in PAH development (5, 6).

Elevated plasma dimeric PKM2 have been detected in various cancer types (2), identifying PKM2 as a potential non-organ-specific biomarker which represents cellular metabolic activity and proliferative capacity. In addition, recent studies have shown metabolic and proliferative abnormalities involving PKM2 in pulmonary hypertension (PH) endothelial cells and fibroblasts (7, 8). PKM2 plasma levels in PH and its expression, localization, phosphorylation, and oligomerization in PAH pulmonary arterial smooth muscle cells (SMCs) remains largely unknown.

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To investigate whether circulating PKM2 serves as a marker for PH, we analyzed the plasma of patients with several PH etiologies from the Giessen PH registry (ethics approval no. 186/16, 266/11) (9). Peripheral plasma from healthy volunteers and central venous plasma from individuals with a mean pulmonary arterial pressure <25 mm Hg (non-PH) served as controls. All participants gave written informed consent. H.G. and H.A.G. had full access to all the data in the study and take responsibility for its integrity and the data analysis. Plasma levels of dimeric PKM2 were significantly reduced in patients with idiopathic PAH (IPAH; n=35) compared with healthy controls (n=30), non-PH controls (n=31), and patients with chronic thromboembolic PH (Group IV PH; n=38) (Figure 1A). The ability of PKM2 to distinguish patients with IPAH from non-PH controls was evaluated using receiver operating characteristic (ROC) analysis. No association of PKM2 plasma with hemodynamic parameters, severity of the disease and survival has been determined.

We next evaluated PKM2 expression, phosphorylation, and oligomerization in lung tissues and/or PASMCs from patients with IPAH and non-PH controls. In accordance with recent findings (7, 8), an elevation of the *PKM2/PKM1* mRNA ratio was noted in the IPAH PASMCs (Figure 1B). PKM2 protein expression was increased in lungs of the patients with IPAH (Figure 1C). Immunofluorescence staining showed that PKM2 expression confined to the medial layer of pulmonary arteries (Figure 1D) and markedly though non-significantly enhanced in PAH-SMCs, compared with non-PH SMCs (Figure 2A). Moreover, we found increased phosphorylation of PKM2 at Y105 in IPAH PASMCs (Figure 2A, 2B). Phosphorylation of Y105 inhibits PKM2 activity by hindering PKM2 tetramer formation (10). The possibility of tetramer/monomer and dimer/monomer formation was analyzed by glutaraldehyde crosslinking assay, which confirmed the formation of dimers (range of 120 kDa) and high molecular weight tetramers (range of 240 kDa) (Figure 2C). Consistent with this and similar to cancer cells, glutaraldehyde cross-linking assays demonstrated a remarkable decay in the assembly of PKM2 tetramers in the lungs and PASMCs of IPAH patients compared with non-PH controls (Figure 2C). Unexpectedly, the decrease in the dimer formation was noted for PAH. The decline in the assembly of low-activity dimers in the PAH-SMCs (Figure 2C) coincides with a marked increase of Y105, but not

S37, phosphorylation of PKM2 (Figure 2D) in PAH-SMCs, suggesting that Y105 phosphorylation contributes to the changes in oligomeric state of the enzyme and thus its activity. We evaluated nuclear PKM2 levels in IPAH and non-PH control PASMCs. IPAH PASMCs showed increased nuclear PKM2 compared with non-PH control PASMCs (Figure 2D), supporting the notion that Y105 phosphorylation may promote PKM2 nuclear translocation to regulate gene transcription.

To understand the potential mechanisms involved in an increase of PKM2 levels, we exposed PASMCs from non-PH controls to hypoxia and platelet derived growth factor (PDGF)-BB, two distinct triggers of abnormal PASMC proliferation. Both hypoxia and PDGF-BB robustly increased Y105 phosphorylation of PKM2, while PDGF-BB further augmented absolute levels of nuclear PKM2 (Figure 2E). A linear dose-dependent relation was observed between nuclear phospho-PKM2 and nuclear markers of G1/S transition, indicating positive correlation between PKM2 elevation and cell proliferation (Figure 2F). Proliferative potential of PAH-SMCs demonstrate clear, but non-significant correlation with PKM2 oligomerization (Figure 2G).

Neither the oligomerization nor the phosphorylation status of PKM2 has been examined in the context of PH and our data add to the recently published findings in PH endothelial cells and fibroblasts, where PKM2 activators, possibly by promoting tetramer formation, reversed the glycolytic phenotype and decreased distinct biosynthetic pathways. Phosphorylation controls the conversion of high-activity tetrameric PKM2 (important for glucose oxidation) to low-activity dimeric/monomeric PKM2 (promotes glycolysis). Thus, reduced PK activity in PH lungs might be partially explained by phosphorylation-dependent allosteric regulation of PKM2. Nuclear translocation of PKM2 in IPAH patient cells does not exclude the existence of other regulatory mechanisms responsible for decline of plasma PKM2. Thus, metabolic reprogramming in PAH may be caused not only by the absolute elevation of PKM2, but also by its allosteric regulation and subsequent nuclear localization (partially affected by phosphorylation). However, it remains unclear which molecular determinants/mechanisms are physiologically responsible for the dissociation of PKM2 dimers into monomers in PAH.

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Disclosures

None

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Figure legend

Figure 1. Plasma levels, expression and localization of PKM2 in PH.

A, Levels of PKM2 in peripheral venous plasma from healthy volunteers and central venous plasma (taken during right heart catheterization) from non-PH controls and patients with PH were determined using a Tu-M2PK Elisa kit (ScheBo Biotech, Giessen, Germany). The area under the ROC curve 0.69. 95% confidence interval 0.56–0.81; *P*<0.01. **B,** Quantitative reverse transcriptase–polymerase chain reaction analyses of mRNA expression of *PKM1* and *PKM2* in PASMCs of patients with IPAH (n=9) versus non-PH controls (n=5). **C,** Quantification of PKM2 in lung tissue from patients with IPAH (n=12) versus non-PH controls (n=11) by immunohistochemistry (Anti-PKM2, ab137791, Abcam, UK) and light microscopy. Scale bars: 50µm. **D,** Representative immunofluorescence images of PKM2 (green) (Anti-PKM2, #3198, CST, USA) and SMA (red) (α-actin, a marker specific for smooth muscle cells, #A2584, Sigma, St Louis, MO) in small pulmonary arteries from human lungs without PAH (non-PH) or with PAH (IPAH). Nuclei are counterstained with DAPI (blue). Scale bars: 50 µm. The images are representative of *n* = 3 lungs per group. CTEPH indicates chronic thromboembolic pulmonary hypertension; DAPI, 4',6-diamidino-2-phenylindole; *HPRT*, hypoxanthine phosphoribosyltransferase; IPAH, idiopathic pulmonary arterial hypertension; n.s., non-significant, and PKM2, pyruvate kinase muscle 2. SMA, α-smooth muscle actin.

Figure 2. Phosphorylation, oligomerization, and regulation of PKM2.

A, Representative Western blot (Phospho-PKM2 [Tyr105] Antibody, #3827, CST, USA and PKM2 Antibody, #3198, CST, USA) and quantification of phospho-PKM2 and total PKM2 in whole cell lysate of PASMCs (non-PH controls, n=5; IPAH, n=8). **B,** Immunofluorescence staining and quantification of phospho-PKM2 in PASMCs (Phospho-PKM2 [Tyr105] Antibody, bs-3334r, Bioss, USA). **C,** Western blot analysis and quantification of PKM2 oligomerization (after crosslinking with glutaraldehyde [0.01%]) in lungs and PASMCs of non-PH controls and patients with IPAH (n=4 for each). **D,** Representative Western blot of nuclear and cytoplasmic phospho-PKM2 (Phospho-PKM2 [Tyr105] Antibody bs-3334r, Bioss, USA, Phospho [Ser37] Antibody, #PA5-37684, Thermo Fischer Scientific, USA) and total PKM2 from non-PH controls and patients with IPAH. Lamin B1 (Anti-Lamin B1, ab90169, Abcam, UK) and α-tubulin (α-tubulin [B-7], sc-5286, Santa Cruz, USA) serve as markers of cytoplasmic and nuclear fractions, respectively. **E,** Representative Western blot and quantification of phospho-PKM2 and total PKM2 in nuclear lysates of non-PH control PASMCs exposed for 24 hours to PDGF-BB and hypoxia. Each column represents three independent experiments. **F,** The correlation between nuclear pPKM2

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and Cyclin E and PCNA was r=0.97 and r=0.96, *P<0.05, respectively. Representative Western blot and quantification of phospho-PKM2, total PKM2, Cyclin E (Cyclin E [E-4], sc-377100, Santa Cruz, USA) and PCNA (PCNA [FL-261], sc-7907, Santa Cruz, USA) in nuclear lysates of non-PH control PASMCs exposed to increasing concentrations of FBS. **G,** Proliferative potential determined by quantification of immunofluorescence staining of Ki67-positive PASMCs exhibit no significant correlation with the tetramer ratio in lungs of patients with IPAH (r=-0.48). P<0.05 was considered statistically significant. Ki67 Antibody (NCL-Ki67p, Novocastra, Leica, Germany) and α-smooth muscle actin (SMA) (α-actin, a marker specific for smooth muscle cells, #A2584, Sigma, St Louis, MO). Human PASMCs (passages 5-7) were seeded and grown at 37°C to 80% confluence for 24 hours. For stimulation studies cells were grown in 60 mm dishes (2×10^5 cells per dish), for oligomerization assays in 100 mm dishes (4×10^5 cells per dish). Prior to PDGF-BB and FBS stimulation, 24 hour serum starvation was performed. For hypoxic experiments cells were cultured at 37°C in hypoxic conditions (5% CO_2 and 1% O_2). To determine statistical significance, one-way analysis of variance followed by Tukey's post-hoc test and unpaired two-tailed Student's t test were utilized. *P<0.05; **P<0.01; ***P<0.001. To determine the correlation in **F** and **G** the Pearson analysis was performed. P<0.05 was considered statistically significant. FBS, fetal bovine serum; GA, glutaraldehyde; PASMCs, pulmonary arterial smooth muscle cells; PDGF-BB, platelet-derived growth factor-BB; PH, pulmonary hypertension; PKM2, pyruvate kinase muscle 2; PCNA, Proliferating cell nuclear antigen; and pPKM2, phospho-pyruvate kinase muscle 2.

Fig. 1

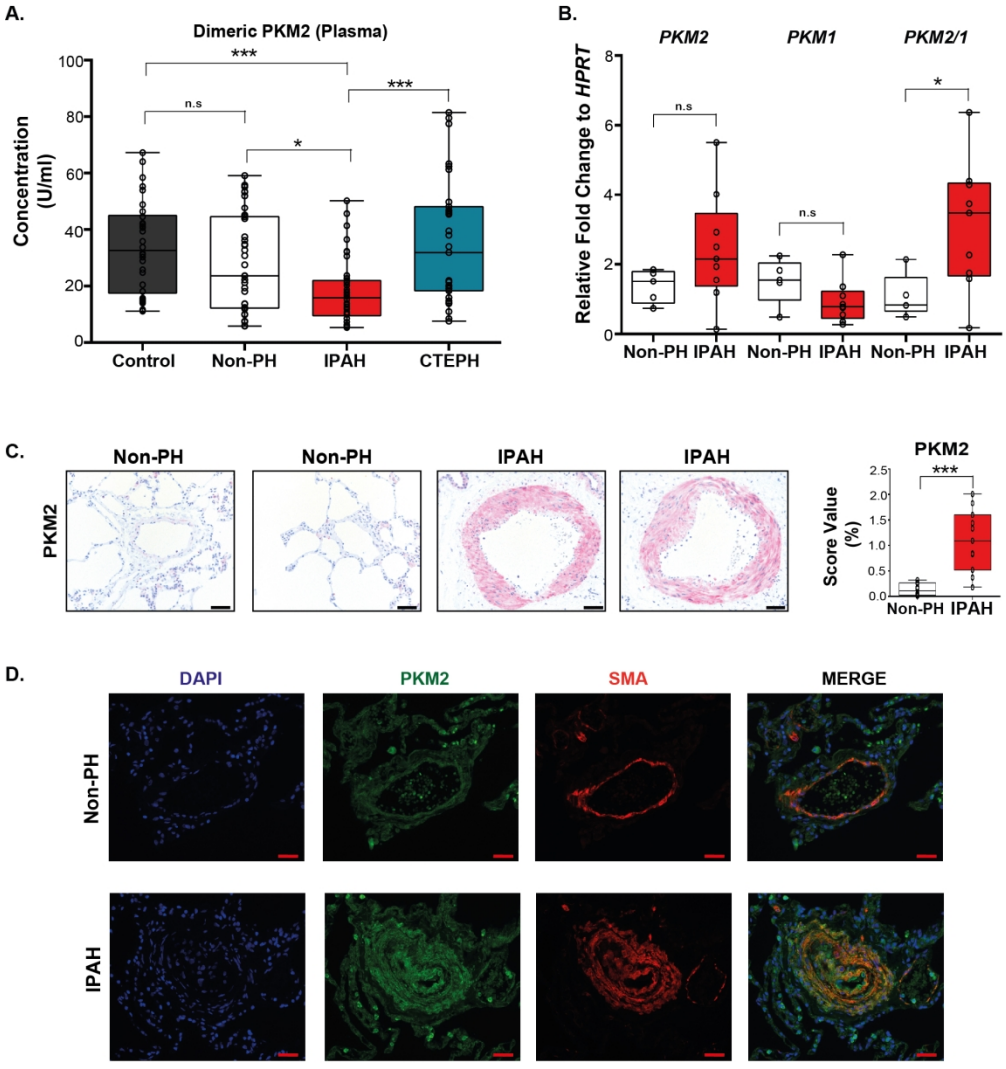

Figure 1. Plasma levels, expression and localization of PKM2 in PH.

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Fig. 2