ORIGINAL RESEARCH

KD025 Shifts Pulmonary Endothelial Cell Bioenergetics and Decreases Baseline Lung Permeability

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Abstract

KD025 is a ROCK2 inhibitor currently being tested in clinical trials for the treatment of fibrotic lung diseases. The therapeutic effects of KD025 are partly due to its inhibition of profibrotic pathways and fat metabolism. However, whether KD025 affects pulmonary microvascular endothelial cell (PMVEC) function is unknown, despite evidence that alveolar–capillary membrane disruption constitutes major causes of death in fibrotic lung diseases. We hypothesized that KD025 regulates PMVEC metabolism, pH, migration, and survival, a series of interrelated functional characteristics that determine pulmonary barrier integrity. We used PMVECs isolated from Sprague Dawley rats. KD025 dose-dependently decreased lactate production and glucose consumption. The inhibitory effect of KD025 was more potent compared with other metabolic modifiers, including 2-deoxy-glucose, extracellular acidosis, dichloroacetate, and remogliflozin. Interestingly, KD025 increased oxidative phosphorylation, whereas 2-deoxy-glucose did not. KD025 also decreased intracellular pH and induced a compensatory increase in anion exchanger 2. KD025 inhibited PMVEC migration, but fasudil (nonspecific ROCK inhibitor) did not. We tested endothelial permeability *in vivo* using Evans Blue dye in the bleomycin pulmonary fibrosis model. Baseline permeability was decreased in KD025-treated animals independent of bleomycin

treatment. Under hypoxia, KD025 increased PMVEC necrosis as indicated by increased lactate dehydrogenase release and propidium iodide uptake and decreased ATP; it did not affect Annexin V binding. ROCK2 knockdown had no effect on PMVEC metabolism, pH, and migration, but it increased nonapoptotic caspase-3 activity. Together, we report that KD025 promotes oxidative phosphorylation; decreases glycolysis, intracellular pH, and migration; and strengthens pulmonary barrier integrity in a ROCK2-independent manner.

Keywords: rho kinase; bleomycin; anion exchanger 2; caspase-3; hypoxia

Clinical Relevance

Prior clinical trials tested the prospect that KD025, a ROCK2 inhibitor, improves fibrosis, although its potential impact on lung endothelium remains unknown. Herein, we report that KD025 inhibits glycolysis, promotes oxidative phosphorylation, inhibits migration, and strengthens barrier integrity in the lung endothelium. Thus, endothelium is an important KD025 drug target that will impact therapeutic efficacy in the treatment of fibrotic lung diseases.

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KD025 (SLx-2119) is a relatively new ROCK2 (ρ [Ras homologous]-associated kinase isoform 2, also known as ROK α) inhibitor (1) currently being tested in multiple phase II clinical trials for the treatment of fibrotic lung diseases, including idiopathic pulmonary fibrosis (ClinicalTrials.gov identifier: NCT02688647) (2), chronic graft versus host disease (ClinicalTrials.gov identifier: NCT03640481), and systemic sclerosis (ClinicalTrials.gov identifier: NCT03919799) related fibrotic diffuse parenchymal lung disease with pulmonary arterial hypertension (3). The therapeutic effect of KD025 is partly via its inhibition of ρ signaling in immune cells (4–6), but the exact mechanism of action is not fully elucidated. KD025 also has a modulatory effect on metabolism by inhibiting adipogenesis in adipocytes, partially in a ROCK2-independent mechanism (7, 8). So far, most studies have been focusing on investigating the therapeutic effects of KD025 in the context of fibrosis, and, therefore, little is known regarding whether it has any effect on pulmonary microvascular endothelial cell (PMVEC) function, which is a critical determinant of alveolar–capillary barrier integrity.

The majority of acutely decompensated deaths in fibrotic lung disease are due to conditions that involve a compromised endothelial barrier, such as acute exacerbation of pulmonary fibrosis and pneumonia (9, 10), illustrating the importance of pulmonary endothelial function in these conditions. Pulmonary endothelial cells isolated from the bleomycin pulmonary fibrosis mouse model possess signs of impaired function, including decreased prostaglandin I_2 and nitric oxide production (11). Pulmonary hypertension significantly increases mortality when it complicates pulmonary fibrosis (PH–PF) (12). However, the pulmonary vasodilators that confer a survival benefit in pulmonary arterial hypertension cannot be used in PF–PH because of their potential harmful effects, which include the generation of ventilation–perfusion mismatching on heterogeneously damaged capillary beds in fibrotic lungs (13, 14). For this reason, inhaled nitric oxide has been tested in heterogeneous lung diseases such as acute respiratory failure syndrome, hoping to induce vasodilation only on the ventilated lung units, but the therapeutic effects have

been inconclusive so far (15). Interestingly, inhaled nitric oxide was shown to improve physical activity in patients with PF–PH in recent updates from an ongoing randomized clinical trial (ClinicalTrials.gov identifier: NCT03267108) (16). Considering the relative vascular selectivity of nitric oxide in its therapeutic effects, the finding further supports the need to better understand pulmonary vascular pathophysiology with additional focus on the alveolar–capillary interface in fibrotic lung diseases.

We have previously described a complex interplay between metabolism, pH, and migration in PMVECs (17). PMVECs are highly glycolytic for their rapid growth (18). Such active glycolysis induces extracellular acidosis, which inhibits PMVEC migration and glycolysis as a negative feedback mechanism. If KD025 has a metabolism-modulating effect on PMVECs, then it is likely that it alters the balance of intracellular and extracellular pH and reduces migration. Here, we investigated how KD025 affects PMVEC metabolism, pH regulation, and migration, a series of critical functional characteristics that determine pulmonary capillary integrity, and we linked these functional characteristics to cell survival and permeability. The findings from this study may offer new insights into the mechanisms of potential therapeutic and adverse effects of KD025 on pulmonary capillary function in fibrotic lung diseases.

Methods

Isolation of Rat Lung Endothelial Cells

Procedures for isolation of rat PMVECs were approved by the University of South Alabama Institutional Animal Care and Use Committee. PMVECs were isolated from male Sprague Dawley rats as previously described (19).

ROCK2 Knockdown

Lentiviral particles containing constructs that encode shRNA designed to knock down ROCK2 gene expression (sc-108088-V) and their control shRNA lentiviral particles (sc-108080) were purchased from Santa Cruz Biotechnology. Cells were transfected with ROCK2 or control shRNA lentiviral particles and selected to homogeneity using puromycin. ROCK2 knockdown was evaluated by Western blot.

Western Blot

Cell lysates were collected and subjected to immunoblot analysis, as previously described (18). Primary antibodies for ROCK2 (160 kD; 1:1,000 dilution), AE2 (anion exchanger 2) (165 kD; 1:200 dilution) and GAPDH (37 kD; 1:1,000 dilution) were obtained from Abcam, Santa Cruz Biotechnology, and Cell Signaling, respectively.

Lactate and Glucose Measurement

Lactate and glucose levels were measured in the media using the YSI 2300 STAT Plus Glucose and Lactate Analyzer (18).

Oxygen Consumption Measurement

Oxygen consumption was measured using the Extracellular O_2 Consumption Assay Kit obtained from Abcam.

Intracellular pH Measurement

Cells were rinsed with Hanks' balanced salt solution and incubated in 1 μ M of 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Thermo Fischer) for 15 minutes. After two rinses with Hanks' balanced salt solution, the intracellular pH was assessed by ratiometric measurements using a plate reader with dual excitation (440 and 490 nm) and single emission (535 nm) wavelengths (20).

Generation of AE2-depleted Cells

PMVECs were transfected with AE2 CRISPR/Cas9 knockout and homology-directed repair plasmids (sc-423008 and sc-423008-HDR; Santa Cruz Biotechnology, respectively) and selected to homogeneity using puromycin. AE2 knockout was evaluated by Western blot.

Scratch-Wound Migration Assay

PMVEC monolayers were scratched with a 200- μ l pipette tip, and baseline wounds were imaged. Then, the media was replaced with bicarbonate-free media and treated with either DMSO (0.15%), KD025 (7.5 μ M), or fasudil (50 μ M). One day after treatments, wounds were reimaged.

Cell-Survival Assays

The Cytotoxicity Detection Assay Kit (Roche Diagnostics), ATP assay kit (AAT Bioquest), propidium iodide (MP Biomedicals), Annexin V FITC (BioLegend), and Caspase-3/CPP32 colorimetric assay kits (Biovision) were used following the manufacturer's instructions.

ρ Kinase Activity Assay

ρ kinase activity was measured on PMVEC lysates following the manufacturer's instructions (Cell Biolabs).

Animals

All experimental procedures were reviewed and approved by the institutional animal care and use committee of the University of South Alabama. F344 rats were bred in house (21).

Rat Bleomycin Pulmonary Fibrosis Model and Lung Permeability Measurements

Bleomycin was introduced into the trachea of male Fischer rats (22) followed by 3 weeks of treatment with either vehicle or KD025 in the drinking water. Evans blue was injected intraperitoneally 1 day before the terminal surgeries. Lung ultrasound and echocardiogram (Vevo 3100) were performed as previously described (23), followed by blood and tissue sampling.

Statistics

One-way ANOVA and Bonferroni *post hoc* tests were used. Significance was denoted as $P < 0.05$.

See the data supplement for greater details.

Results

KD025 Inhibits PMVEC Glycolysis in a Dose-Dependent Manner

We have previously reported that glycolytic pathway modifiers affect PMVEC movement. 2DG has no effect on migration but decreases network formation, whereas extracellular acidosis decreases migration and increases network formation in PMVECs (17). ρ kinase activity is important for cell migration (24, 25), but its impact on bioenergetics is unclear. To address this issue, we investigated the effect of KD025 on PMVEC glycolysis by measuring lactate production and glucose consumption. PMVECs were glycolytically active at baseline (Figures 1A and 1B), as we have previously reported (17, 18, 20, 26, 27). KD025 decreased lactate production (Figure 1A; DMSO vs. KD025 750 nM, KD025 7.5 μ M, and KD025 15 μ M; $P < 0.05$) and glucose consumption (Figure 1B; DMSO vs. KD025 7.5 μ M and KD025 15 μ M; $P < 0.05$) in a dose-dependent manner. The inhibitory effect of

KD025 on PMVEC glycolysis was most prominent at micromolar concentrations, wherein an adipogenesis modulating effect of KD025 has been reported by others (8).

KD025 Potently Inhibits PMVEC Glycolysis

To further characterize mechanisms involved in KD025-induced glycolysis inhibition, we compared the effect of KD025 with other known metabolism modifiers, including 2DG (competitive glycolysis inhibitor) (28), extracellular acidosis (17), dichloroacetate (DCA) (pyruvate dehydrogenase kinase inhibitor) (29), and remogliflozin (sodium glucose cotransporter 2 inhibitor) (30), on extracellular and intracellular lactate and glucose. A range of concentrations have been tested for 2DG, media pH, DCA, and remogliflozin, and the concentrations that induce significant metabolic alterations with minimal cytotoxicity on gross microscopic exam were determined based on preliminary studies (data not shown).

Consistent with the known mechanism as a competitive inhibitor of glucose (28), 2DG decreased glycolysis indicated by decreased extracellular lactate production (Figure 2A; DMSO vs. 2DG; $P < 0.05$) and intracellular lactate production (Figure 2B; DMSO vs. 2DG; $P < 0.05$) in PMVECs. Extracellular glucose was higher (Figure 2C; DMSO vs. 2DG; $P < 0.05$) and intracellular glucose was unchanged (Figure 2C; DMSO vs. 2DG, $P =$ not significant [ns]) with 2DG; however, because 2DG and glucose are indistinguishable in this assay, the 2DG effect on glucose level was not accurately measured.

We previously showed that extrinsic acidosis inhibits PMVEC glycolysis and that such an inhibitory effect can be reversed by extrinsic alkalization (17). In the current study, pH 6.8 decreased extracellular (Figure 2A; DMSO vs. pH 6.8; $P < 0.05$) and intracellular (Figure 2B; DMSO vs. pH 6.8; $P < 0.05$) lactate and attenuated the decrease in extracellular glucose (Figure 2C; DMSO vs. pH 6.8; $P < 0.05$) without changing intracellular glucose (Figure 2D; DMSO vs. pH 6.8; $P =$ ns). The data confirm our previous finding that extrinsic acidosis inhibits PMVEC glycolysis.

DCA inhibits pyruvate dehydrogenase kinase and shifts the metabolic pathway from glycolysis to oxidative phosphorylation (29). In our study, DCA decreased extracellular (Figure 2A; DMSO

vs. DCA; $P < 0.05$) and intracellular (Figure 2B; DMSO vs. DCA; $P < 0.05$) lactate in PMVECs. However, in contrast to 2DG or pH 6.8, DCA did not affect extracellular glucose (Figure 2C; DMSO vs. DCA; $P =$ ns), although it decreased intracellular glucose (Figure 2D; DMSO vs. DCA; $P < 0.05$). Together with decreased lactate production, this finding suggests that DCA decreased glycolysis and increased transcellular glucose transport in PMVECs.

Remogliflozin is a sodium glucose cotransporter 2 inhibitor that is currently used in the treatment of type 1 diabetes, in which it enhances renal tubular epithelial glucose excretion (30). Interestingly, remogliflozin increased extracellular lactate (Figure 2A; DMSO vs. remogliflozin; $P < 0.05$) and intracellular glucose consumption (Figure 2D; DMSO vs. remogliflozin; $P < 0.05$) without eliciting a change in intracellular lactate (Figure 2B; DMSO vs. remogliflozin; $P =$ ns) or extracellular glucose (Figure 2C; DMSO vs. remogliflozin; $P =$ ns). Therefore, remogliflozin likely increases, rather than decreases, glycolysis.

Among all the drugs tested, KD025 was the most potent modifier of PMVEC glycolysis, considering that it elicited the greatest degree of lactate and glucose changes at the lowest concentrations (Figures 2A–2D). Known mechanisms of action of the metabolic modifiers are summarized in Figure 2E.

KD025 Inhibits Glycolysis and Increases Oxidative Phosphorylation in PMVECs in a ROCK2-Independent Manner

To assess whether the effect of KD025 on PMVEC glycolysis is ROCK2 mediated, we generated ROCK2-knockdown cells using shRNA. Figure 3A shows the successful knockdown of ROCK2 in PMVECs. Interestingly, neither lactate production (Figure 3B; control vs. ROCK2 shRNA DMSO; $P =$ ns) nor glucose consumption (Figure 3C; control vs. ROCK2 shRNA DMSO; $P =$ ns) were affected by ROCK2 knockdown. The inhibitory effect of KD025 on lactate production and glucose consumption was consistently shown in control (Figure 3B; control shRNA DMSO vs. KD025; $P < 0.05$) and ROCK2-knockdown (Figure 3C; ROCK2 shRNA DMSO vs. KD025; $P < 0.05$) PMVECs. Fasudil had no effect on glycolysis (Figures

Figure 1. KD025 inhibits pulmonary microvascular endothelial cell (PMVEC) glycolysis in a dose-dependent manner. PMVECs were seeded at 5.0×10^5 cells/well on 6-well plates in bicarbonate-buffered media. Two days after cell seeding, media was changed to HEPES-buffered media with a pH of 7.4 and treated with either 0.15% DMSO or a range of concentrations of KD025 (75 nM–15 μM). (A and B) Twenty-four hours later, media was collected for lactate (A) and glucose measurements (B). KD025 decreased lactate production and glucose consumption in a dose-dependent manner. Data represent mean $\pm$ SD. Two-way ANOVA and Bonferroni *post hoc* tests were used to compare different groups. At least five separate experiments were performed. *Significant difference ($P < 0.05$) from DMSO.

3B and 3C; control shRNA DMSO vs. fasudil; $P = 0.05$). Therefore, KD025 inhibits PMVEC glycolysis in a ROCK2-independent manner.

In addition to its effect on glycolysis, KD025 increased oxygen consumption (Figure 3D; control shRNA DMSO vs. KD025; $P < 0.05$), whereas fasudil did not (Figure 3D; control shRNA DMSO vs. fasudil; $P = \text{ns}$). To assess whether this metabolic shift from glycolysis to oxidative phosphorylation is a general PMVEC compensatory phenomenon in response to glycolysis inhibitors, we tested high-dose 2DG (100 mM). Unlike KD025, 2DG had no effect on oxygen consumption (Figure 3D; control shRNA DMSO vs. 2DG; $P = \text{ns}$). This KD025 effect on oxygen consumption was not mediated by ROCK2 (Figure 3D; control vs. ROCK2 shRNA DMSO; $P = \text{ns}$). Therefore, KD025 shifts metabolic pathways from glycolysis to oxidative phosphorylation in a ROCK2-independent manner in PMVECs.

KD025 Decreases Intracellular pH and Increases AE2 in PMVECs in a ROCK2-Independent Manner

Metabolism is a major source of protons. However, changes in metabolism do not necessarily alter intracellular pH because multiple layers of pH regulators, including buffering systems and transcellular proton transporters, jointly operate to maintain pH homeostasis (31). It is not known whether KD025, which induces a metabolic shift from glycolysis to oxidative phosphorylation, affects intracellular pH in

PMVECs. We first measured culture media pH, which was higher with KD025 treatment (Figure 4A; control shRNA DMSO vs. KD025; ROCK2 shRNA DMSO vs. KD025; $P < 0.05$). Next, using a pH-sensitive BCECF dye, we found that KD025 significantly decreases PMVEC intracellular pH (Figure 4B; control shRNA DMSO vs. KD025; $P < 0.05$) in a ROCK2-independent manner (Figure 4B; control vs. ROCK2 shRNA DMSO; $P = \text{ns}$). Interestingly, this KD025-induced decrease in intracellular pH was accompanied by increases in AE2 protein abundance (Figures 4C and 4D; control shRNA DMSO vs. KD025; $P < 0.05$). However, AE2 knockout (Figure 4E) had no effect on the KD025-induced shifts in extracellular and intracellular pH, lactate, and glucose (Figures 4F–4I; AE2 knockout DMSO vs. KD025; $P < 0.05$), suggesting that the increase in AE2 is a compensatory response to the altered transcellular proton gradient. Fasudil had no effect on pH or AE2 (Figures 4B–4D; control shRNA DMSO vs. fasudil; $P = \text{ns}$).

KD025 Inhibits PMVEC Migration in a ROCK2-Independent Manner

Having identified the regulatory roles of KD025 on PMVEC metabolism and intracellular pH, we further assessed its effect on migration, which is an important determinant of wound healing and barrier integrity. The scratch-wound migration assay revealed an inhibitory effect of KD025 on PMVEC migration (Figures 5A–5D; control shRNA DMSO vs. KD025;

$P < 0.05$), in a ROCK2-independent manner (Figures 5A–5D; control vs. ROCK2 shRNA DMSO; $P = \text{ns}$). Fasudil had no effect on PMVEC migration (Figures 5A–5D; control shRNA DMSO vs. fasudil; $P = \text{ns}$). Therefore, the KD025-mediated decrease in glycolysis and increase in oxidative phosphorylation are accompanied by a reduced migratory capacity, all of which are independent of ROCK2.

KD025 Decreases Pulmonary Endothelial Permeability

So far, we have demonstrated that KD025 affects PMVEC bioenergetics, extracellular and intracellular pH, and migratory capacity. These functional characteristics of endothelial cells are critically important in maintaining pulmonary vascular integrity. Therefore, we measured pulmonary endothelial permeability using an Evans blue dye assay in a bleomycin pulmonary fibrosis Fischer rat model. Bleomycin was introduced in a single intratracheal instillation, followed by 3 weeks of vehicle or KD025 treatment via the drinking water. Bleomycin treatment induced local lung inflammation, which was detected by lung ultrasound (Figure 6A) and histology (Figure 6B). However, unlike multiple murine studies that reported increased lung permeability and pulmonary hypertension after bleomycin challenge (32–34), our animals did not develop a significant increase in lung permeability or pulmonary hypertension (data not shown) at this time point. However, KD025 still decreased baseline lung permeability independent of bleomycin injury (Figure 6C; vehicle vs. KD025; $P < 0.05$), suggesting a barrier-enhancing effect of the drug on pulmonary endothelium *in vivo*.

KD025 Causes Necrotic Death during a Hypoxia Exposure, and ROCK2 Inhibits Nonapoptotic Caspase-3 Activity in PMVECs

Patients with severe lung diseases commonly present with hypoxemia. Whether a global effect of KD025 on metabolism, pH and migration collectively contributes to PMVEC survival in normoxic and hypoxic environments is a clinically relevant question. To address this issue, we measured lactate dehydrogenase (LDH) release and propidium iodide uptake as markers of necrotic cell death, ATP as a marker of survival, and caspase-3 activity

Figure 2. KD025 potentially inhibits PMVEC glycolysis. PMVECs were seeded at 5.0×10^5 cells/well on 6-well plates in bicarbonate-buffered media. Two days after cell seeding, the media was changed to HEPES-buffered media with a pH of 7.4 and treated with DMSO (0.15%), KD025 (7.5 μ M), HCl (to achieve pH 6.8), 2-deoxy-glucose (10 mM), dichloroacetic acid (25 mM), and remogliflozin (7.5 μ M). (A–D) Twenty-four hours later, media (A and C) and whole-cell lysates (B and D) were collected for lactate and glucose measurements. KD025 most effectively decreased lactate production and glucose consumption at the lowest drug concentration. Mechanisms of metabolic modifiers in comparison with KD025 are schematically illustrated in E. Data represent mean $\pm$ SD. Two-way ANOVA and Bonferroni *post hoc* tests were used to compare between different groups. At least five separate experiments were performed. *Significant difference ($P < 0.05$) from DMSO. Plus sign indicates treated and minus sign indicates not treated. 2DG = 2-deoxy-glucose; DCA = dichloroacetate; HXK I = hexokinase I; PDH = pyruvate dehydrogenase; PDK = PDH kinase; SGLT2 = sodium-glucose co-transporter 2.

Figure 3. KD025 inhibits glycolysis and increases oxidative phosphorylation in PMVECs in a ROCK2 (ρ [Ras homologous]-associated kinase isoform 2)-independent manner. PMVECs were transfected with control or ROCK2 shRNA. (A) ROCK2 knockdown was confirmed by whole-cell lysate Western blot. (B–D) Control and ROCK2-knockdown PMVECs were seeded at 5.0×10^5 cells/well on 6-well plates (B and C) and at 3.0×10^4 cells/well on 96-well plates in bicarbonate-buffered media (D). Two days after cell seeding, the media was changed to HEPES-buffered media with a pH of 7.4 and treated with DMSO (0.15%), KD025 (7.5 μ M), fasudil (50 μ M), and 2DG (100 mM, for oxygen consumption assay). (B–D) Twenty-four hours later, media lactate (B), media glucose (C), and cellular oxygen consumption (D) were measured. KD025 decreased glycolysis and increased oxygen consumption in a ROCK2-independent manner. Data represent mean $\pm$ SD. Two-way ANOVA and Bonferroni *post hoc* tests were used to compare different groups. At least five separate experiments were performed. *Significant difference ($P < 0.05$) from control shRNA DMSO. Plus sign indicates treated and minus sign indicates not treated.

and annexin V binding as markers of apoptosis in PMVECs in both normoxic and hypoxic conditions. KD025 had a minimal effect on LDH release, propidium iodide uptake, or ATP in normoxia (Figures 7A–7C; normoxia control shRNA DMSO vs. normoxia control shRNA KD025; $P = \text{ns}$), but hypoxia profoundly increased LDH release and propidium iodide uptake and decreased ATP (Figures 7A–7C; normoxia control shRNA KD025 vs. hypoxia control shRNA KD025; $P < 0.05$). Interestingly, ROCK2 knockdown, which had no effect on any other phenotypes we investigated in this study, significantly increased caspase-3 activity at baseline (Figure 7D; normoxia control shRNA DMSO vs. normoxia ROCK2 shRNA DMSO; $P < 0.05$). However, annexin V binding, a specific marker of apoptosis, was not different across all groups (Figure 7E),

suggesting a potential that nonapoptotic caspase-3 activity was increased by both ROCK2 knockdown and hypoxia. To address the discrepancy between the effect of KD025 and ROCK2 knockdown on caspase-3 activity, we measured Rho kinase activity, which assesses both ROCK1 and ROCK2 activity. KD025 treatment led to only a minimal suppression of ρ kinase activity (Figure 7F; control shRNA DMSO vs. control shRNA KD025, $P = \text{ns}$), but a significant suppression of enzymatic activity was observed following ROCK2 knockdown (Figure 7F; control shRNA DMSO vs. ROCK2 shRNA DMSO; $P < 0.05$). Fasudil also increased caspase-3 activity in normoxic conditions (Figure 7D; normoxia control shRNA DMSO vs. normoxia control shRNA fasudil; $P = \text{ns}$), but there was no additional increase under hypoxia (Figure 7D; normoxia control

shRNA fasudil vs. hypoxia control shRNA fasudil; $P = \text{ns}$). Therefore, KD025 confers necrosis in hypoxic conditions, and ROCK2 suppresses the nonapoptotic caspase-3 activity in PMVECs.

Discussion

In this study, we investigated the effect of KD025 on PMVEC metabolism, pH, migration, and survival. We report the following four key findings: 1) KD025 shifts PMVEC metabolic pathways from glycolysis to oxidative phosphorylation and decreases intracellular pH and migration in a ROCK2-independent manner; 2) KD025 strengthens the pulmonary endothelial barrier integrity *in vivo*; 3) KD025 causes PMVEC necrosis in hypoxia; and 4) ROCK2 inhibits nonapoptotic caspase-3 activity in PMVECs. Our findings suggest that KD025 drives the PMVEC metabolic, pH, migratory, and survival phenotype to one that tightens barrier integrity in normoxic conditions, whereas it potentially increases susceptibility to injuries when exposed to hypoxic conditions. The key findings are summarized in Figure 7G.

PMVECs primarily use glycolysis even in the presence of abundant oxygen (17, 18, 20, 26, 27). This phenomenon is called the Warburg effect, which was first described in cancer cells (35, 36). PMVECs are cancer-like (37) regarding their high glycolytic activity and excellent proliferative potential (26). The advantage of PMVECs maintaining such a metabolic phenotype is believed to be multifold, including preserving oxygen for its use by tissue parenchymal cells and rapidly producing the ATP and biomass required to support proliferation while concomitantly cycling through the pentose phosphate pathway (38). In our study, we demonstrated that KD025 profoundly inhibits PMVEC glycolysis. The inhibitory effect was dose dependent, and it became prominent at a micromolar concentration range. This dose range is where Diep and colleagues reported an adipogenesis modulatory effect in adipocytes (8), and it is the concentration at which the original drug developers reported cytoskeletal changes in pulmonary arterial smooth muscle cells (1). Other metabolic modifiers, including 2DG and DCA, required millimolar concentration to induce lesser degrees of extracellular and intracellular lactate and

Figure 4. KD025 decreases intracellular pH and increases AE2 (anion exchanger 2) in PMVECs in a ROCK2-independent manner. Control and ROCK2-knockdown PMVECs were seeded at 5.0×10^5 cells/well on 6-well plates in bicarbonate-buffered media. Two days after cell seeding, the media was changed to HEPES-buffered media with a pH of 7.4 and treated with DMSO (0.15%), KD025 (7.5 μ M), and fasudil (50 μ M). (A and B) KD025 prevented media acidification (A) and decreased intracellular pH (B). (C and D) KD025 increased AE2 protein abundance in cell lysates measured by Western blot. (E) AE2-knockout PMVECs were generated using the CRISPR/Cas9 technique. (F–I) AE2-knockout cells showed the same response to KD025, including increased extracellular pH (F), decreased intracellular pH (G), decreased lactate (H), and increased glucose changes as WT PMVECs (I). Data represent mean $\pm$ SD. Two-way ANOVA and Bonferroni *post hoc* tests were used to compare different groups. At least five separate experiments were performed. *Significant difference ($P < 0.05$) from control shRNA DMSO. Plus sign indicates treated and minus sign indicates not treated. K/O = knockout; WT = wild type.

glucose changes, indicating greater potency of KD025 on PMVEC glycolysis inhibition.

Glycolysis and oxidative phosphorylation are complementary, and they are flexibly used by cells to support metabolic needs. An increase in the rate of glycolysis with suppressed oxidative phosphorylation occurs in response to diverse pathological stimuli involving tissue hypoxia, infection, and malignancy (35, 36, 39, 40). However, relatively little is known regarding whether the inhibition of glycolysis enhances oxidative

phosphorylation. Two studies have reported such a “Warburg reversing effect” in cancer cells, in which hexokinase II inhibition decreases glycolysis and, at the same time, increases oxidative phosphorylation (41, 42). Investigators proposed this increased oxidative phosphorylation in the setting of inhibited glycolysis serves as an escape mechanism in cancer cells to resist anticancer therapy. Robinson and colleagues reported a similar phenomenon in which cancer cells shift metabolism from glycolysis to oxidative

phosphorylation in glucose-free media to become less sensitive to intrinsic cell-death stimuli (ABT-737 and ionizing radiation) (43). In our study, we observed that KD025 induced a metabolic pathway shift from glycolysis to oxidative phosphorylation in PMVECs. This KD025 effect was particularly interesting when compared with the effect of 2DG. KD025 is much more potent than 2DG, and it takes over a ten thousand-fold higher concentration of 2DG to achieve comparable glycolysis inhibition (data not shown). Even when the

Figure 5. KD025 inhibits PMVEC migration in a ROCK2-independent manner. Control and ROCK2-knockdown PMVECs were seeded at 5.0×10^5 cells/well on 6-well plates in bicarbonate-buffered media. Two days after cell seeding, monolayers were scratched with a sterile 200- μ l pipette tip. The media was changed to HEPES-buffered media with a pH of 7.4 and treated with DMSO (0.15%), KD025 (7.5 μ M), and fasudil (50 μ M). (A–D) Wounds were imaged and analyzed using ImageJ at baseline (A and B) and 24-hour time points (C and D). KD025 decreased migration in a ROCK2-independent manner. Fasudil had no effect on PMVEC migration. Data represent mean $\pm$ SD. Two-way ANOVA and Bonferroni *post hoc* tests were used to compare between different groups. At least five separate experiments were performed. *Significant difference ($P < 0.05$) from control shRNA DMSO. Plus sign indicates treated and minus sign indicates not treated. Scale bar, 200 μ g.

degree of glycolysis inhibition is matched by increasing the 2DG concentration, unlike KD025, 2DG does not increase oxidative phosphorylation, indicating a distinctive mechanism is involved in the KD025-induced shift in metabolism. Overall, the metabolism-modulating effect of KD025 has a number of potential clinical applications. If KD025 has similar effects on activated fibroblasts, which possess enhanced glycolysis (44), the shift to oxidative phosphorylation may be one of the mechanisms contributing to its antifibrotic effect on fibrotic lung diseases.

The KD025-induced increase in oxidative phosphorylation in PMVECs may have helpful or harmful effects depending on the status of modulating factors, such as oxygen availability and underlying mitochondrial function. Future studies addressing this issue will offer insights into the efficacy and potential side effects of KD025.

Glycolysis is advantageous for rapidly proliferating cells, as discussed above. However, it is accompanied by a burden of increased proton production, which can compromise pH homeostasis. Septic shock is a relevant example of excessive glycolysis

that subsequently results in rapid progression of acidosis and hemodynamic instability (45). During such enhanced glycolysis, cytosolic proton concentration increases. However, cells maintain pH homeostasis by using redundant acid handling mechanisms, including buffers, channels, and transporters (46). The capacity of acid handling mechanisms varies among different cell types (47), and PMVECs are one of the cells with a high acid handling capacity. PMVECs use carbonic anhydrase IX as a major contributor to their buffer system (20). In

Figure 6. KD025 decreases baseline pulmonary permeability. Fischer rats were tracheally instilled with PBS or bleomycin, followed by 3 weeks of either vehicle (0.4% methylcellulose) or KD025 (5.25 mg/d/animal) treatments. Representative lung ultrasound and histology images are shown in A and B. (A and B) Bleomycin induced local lung disease indicated by focal B-lines on lung ultrasound (A) and areas of inflammation and fibrosis on hematoxylin and eosin staining of the lung (B). Scale bar, 200 μ m. (C) KD025 treatment decreased Evans blue extravascular leakage across all treatment groups independently of bleomycin challenge status. B-lines are hyperechoic vertical lines obscuring normal A-lines (hyperechoic regularly spaced horizontal repetitive lines, as shown in PBS lung). Data represent mean $\pm$ SD. Student's *t* test was used to compare two groups. *Significant difference ($P < 0.05$) from vehicle.

our current study, we report that KD025 significantly decreases PMVEC intracellular pH at 24 hours. This was a striking finding, because we have previously demonstrated that PMVECs possess a remarkable ability to maintain their intracellular pH with exposure to a wide range of extracellular pHs (17). Furthermore, decreased, rather than increased, intracellular pH was counterintuitive, considering that profound glycolysis suppression by KD025 would decrease the cytosolic proton burden. Interestingly, KD025 significantly increased AE2 protein abundance, which seems to be a compensatory mechanism to adjust extracellular and intracellular pH.

Migration is an important functional characteristic of PMVECs for their maintenance of barrier integrity and facilitation of wound healing. ρ kinases contribute to endothelial barrier function via their regulation of myosin light-chain

kinase phosphorylation and actomyosin interaction (48). In our study, KD025 suppressed PMVEC migration, but it was in a ROCK2-independent manner. At first glance, the lack of a ROCK2-specific role in PMVEC migration seems contradictory to the findings from previous studies (49–52). However, unlike our experimental setting, in most studies reporting a ROCK2-specific role in migration and barrier function, the enzyme was inhibited in the presence of inflammatory or hypoxic conditions. Therefore, we believe our results represent either a direct chemical inhibition of KD025 on the PMVEC migratory machinery or alternatively, an indirect regulation of migration through a second hit from the KD025-induced metabolic and intracellular pH shifts.

The involvement of ρ kinases in apoptotic pathways has been reported, but a majority of the findings were based on

nonspecific chemical (fasudil and Y27632) or genetic (dominant negative mutants of ROCK) inhibition (53, 54). Studies using genetic approaches generally proposed proapoptotic roles of ROCK1 and ROCK2. For example, a study using ROCK1-knockout mice showed an important role of ROCK1 in activating proapoptotic caspase-3 (55). Studies using ROCK2 overexpression (56) and knockdown (57) approaches *in vitro* showed that ROCK2 activity is linked to apoptotic cellular morphological changes and proapoptotic signaling. We report an inhibitory role of ROCK2 on nonapoptotic caspase-3 activity in PMVECs. Whether nonapoptotic caspase-3 activity has physiologic consequences, such as a permeability protective role (58) in the context of ROCK2 signaling, requires additional studies. The KD025 effect on necrotic cell death was minimal under normoxia, but it was dramatically increased under hypoxia. Considering that the KD025-induced metabolic shift from glycolysis to oxidative phosphorylation persists under hypoxia (data not shown), we speculate that a compromise in oxygen with profoundly suppressed glycolysis may result in insufficient ATP production and cell death. Because apoptosis requires ATP (59), necrosis would be a predominant mechanism of cell death in oxidative phosphorylation-dependent metabolic conditions.

Clinical implications of such a hypoxia effect on enhancing KD025 toxicity can be variable. Depending on the context, hypoxia may accelerate the therapeutic effect of KD025 on pathologically rapidly growing cells, but at the same time, it may result in toxicity on normally untargeted cells. Although our animal study revealed that KD025 reduces baseline lung permeability, the currently used bleomycin pulmonary fibrosis model was not severe enough to cause global endothelial barrier injury that either increases Evans blue leakage or induces echocardiographic signs of pulmonary hypertension. Therefore, increasing the bleomycin concentration and/or combining it with a concurrent hypoxic challenge in future studies may be required to identify severe consequences of the KD025 effect on endothelial barrier integrity (32).

In summary, herein, we report that KD025, a drug that has been mainly studied in the context of fibrotic diseases, has multiple effects on PMVEC function. KD025

Figure 7. KD025 causes necrotic death under hypoxia, and ROCK2 inhibits nonapoptotic caspase-3 activity in PMVECs. Control and ROCK2-knockdown PMVECs were seeded at 5.0×10^5 cells/well on 6-well plates in bicarbonate-buffered media. Two days after cell seeding, the media was changed to HEPES-buffered media with pH 7.4 and treated with either DMSO (0.15%), KD025 (7.5 μ M), or fasudil (50 μ M), and the cells were incubated in either normoxia or hypoxia (1% O_2). Twenty-four hours later, media, cells, and lysates were used for lactate dehydrogenase release (media), propidium iodide uptake (cells), ATP (cells), caspase-3 activity (lysates), and annexin V binding (cells) measurements. (A–C) KD025 and concomitant hypoxia exposure induced an extensive increase in lactate dehydrogenase release (A) and propidium iodide uptake (B) while also decreasing ATP (C), indicating increased necrotic cell death. (D and E) ROCK2 knockdown, but not KD025, increased caspase-3 activity (D) without affecting annexin V binding (E), suggesting an inhibitory role of ROCK2 on nonapoptotic caspase-3. (F) ROCK activity was significantly decreased by ROCK2 knockdown but not by KD025, confirming the ROCK2-independent KD025 effect on metabolism, pH, and migration. A schematic representation is shown in G. KD025 has a global effect on PMVEC metabolism, pH, migration, and survival, and it decreases lung permeability. KD025 potentially decreased glycolysis and increased oxidative phosphorylation. Despite suppressed glycolysis and presumably decreased proton production, intracellular pH was decreased, which increases AE2 abundance. KD025 decreased migration in a ROCK2-independent manner, as metabolic and pH changes were independent of ROCK2. KD025 had a minimal effect on cell necrosis under normoxia, but it massively increased necrosis under hypoxia. KD025 decreased lung permeability. ROCK2 inhibited nonapoptotic caspase-3 activity. Data represent mean $\pm$ SD. Two-way ANOVA and Bonferroni *post hoc* tests were used to compare different groups. At least five separate experiments were performed. *Significant difference ($P < 0.05$) from normoxia control shRNA DMSO. ^Significant difference ($P < 0.05$) from normoxia control shRNA KD025. Plus sign indicates treated and minus sign indicates not treated. LDH=lactate dehydrogenase.

increases oxidative phosphorylation and decreases aerobic glycolysis, intracellular pH, and migration while upregulating AE2 in PMVECs, all in a ROCK2-independent manner. KD025 confers significant cytotoxicity under hypoxia, and ROCK2

inhibits nonapoptotic caspase-3 activity. KD025 enhances baseline rat lung barrier integrity, for which future studies are warranted to elucidate clinical implications in the context of pathologic conditions. Our findings offer novel mechanistic insights into

the therapeutic effects of KD025 on fibrotic lung diseases and may help identify new therapeutic applications of this compound. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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