Glc-6-PD and PKG contribute to hypoxia-induced decrease in smooth muscle cell contractile phenotype proteins in pulmonary artery

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Chettimada S, Rawat DK, Dey N, Kobelja R, Simms Z, Wolin MS, Lincoln TM, Gupte SA. Glc-6-PD and PKG contribute to hypoxia-induced decrease in smooth muscle cell contractile phenotype proteins in pulmonary artery. Am J Physiol Lung Cell Mol Physiol 303: L64–L74, 2012. First published May 11, 2012; doi:10.1152/ajplung.00002.2012.—Persistent hypoxic pulmonary vasoconstriction (HPV) plays a significant role in the pathogenesis of pulmonary hypertension, which is an emerging clinical problem around the world. We recently showed that hypoxia-induced activation of glucose-6-phosphate dehydrogenase (Glc-6-PD) in pulmonary artery smooth muscle links metabolic changes within smooth muscle cells to HPV and that inhibition of Glc-6-PD reduces acute HPV. Here, we demonstrate that exposing pulmonary arterial rings to hypoxia (20–30 Torr) for 12 h in vitro significantly (P < 0.05) reduces (by 30–50%) SM22α and smooth muscle myosin heavy chain expression and evokes HPV. Glc-6-PD activity was also elevated in hypoxic pulmonary arteries. Inhibition of Glc-6-PD activity prevented the hypoxia-induced reduction in SM22α expression and inhibited HPV by 80–90% (P < 0.05). Furthermore, Glc-6-PD and protein kinase G (PKG) formed a complex in pulmonary artery, and Glc-6-PD inhibition increased PKG-mediated phosphorylation of VASP (p-VASP). In turn, increasing PKG activity upregulated SM22α expression and attenuated HPV evoked by Glc-6-PD inhibition. Increasing passive tension (from 0.8 to 3.0 g) in hypoxic arteries for 12 h reduced Glc-6-PD, increased p-VASP and SM22α levels, and inhibited HPV.

The present findings indicate that increases in Glc-6-PD activity influence PKG activity and smooth muscle cell phenotype proteins, all of which affect pulmonary artery contractility and remodeling.

GENERALIZED PERSISTENT HYPOXIA is a major cause of pulmonary hypertension (PH) in patients with chronic obstructive pulmonary disease and in individuals living at high altitude (45). Over the years, some studies have suggested that chronic hypoxia-induced PH is mainly the result of increased pulmonary artery constriction, whereas other studies have suggested that remodeling of the pulmonary artery increases resistance, thereby contributing to PH. Both these factors, along with inflammation, are now considered to be important causes of PH (45). However, the molecular mechanisms and signaling pathways involved in hypoxia-induced pulmonary arterial constriction and remodeling during the pathogenesis of PH are not completely understood, and an effective treatment for PH is still lacking (11).

Increases in Ca²⁺ influx through L-type or store-operated Ca²⁺ channels, Ca²⁺ release from the internal stores, and increased Ca²⁺ sensitivity of the myofilaments have all been implicated in hypoxia-induced pulmonary vasoconstriction (21). It is now clear that increased Rho kinase activity plays a key role in enhancing the Ca²⁺ sensitivity of the myofilaments and in pulmonary arterial constriction in hypoxia- and monocrotaline-induced PH and in sugen5416 (a VEGF receptor blocker)-hypoxia-induced pulmonary arterial hypertension (35). It has been speculated that hypoxia-induced increases in circulating neurohumoral factors, cytokines, and autacoids stimulate G-protein-coupled receptors that activate RhoA-Rho kinase signaling and evoke pulmonary arterial contraction (29). In addition, hypoxic factors, including Rho kinase, also elicit remodeling of the small (50–100 μm) pulmonary arteries. In hypoxia-induced PH, thickening of the pulmonary arterial media reduces luminal cross-sectional area, thereby decreasing blood flow. By contrast, in pulmonary arterial hypertension, formation of neointimal and plexiform lesions occludes the pulmonary artery (45). During remodeling, pulmonary arterial endothelial cell size is increased, the smooth muscle phenotype is modulated from the contractile to the synthetic phenotype, and inflammatory and progenitor stem cells are recruited. All of these processes contribute to remodeling of the pulmonary vasculature (38). However, the signaling mechanisms between O₂ sensor and effector molecule have not yet been established in pulmonary artery smooth muscle (PASM). Moreover, it is not known how hypoxia causes phenotypic modulation of PASM from the contractile to the synthetic phenotype.

Our laboratory recently showed that hypoxia-induced increases in glucose-6-phosphate dehydrogenase (Glc-6-PD) activity and NADPH-to-NADP⁺ ratios in PASM are a temporal link between metabolic changes within cells and hypoxic pulmonary vasoconstriction (HPV) and that inhibition of Glc-6-PD reduces HPV (15, 19, 20). However, it remains unclear how increased Glc-6-PD activity evokes HPV, and we believe elucidating that process could lead to the development of new strategies to prevent or reverse PH in the future. For instance, we have shown that a competitive Glc-6-PD inhibitor, 6-amino nicotinamide (6AN), and two uncompetitive Glc-6-PD inhibitors, epiandrosterone and dehydroepiandrosterone, evoke relaxation of pulmonary and coronary arteries and reduce HPV by decreasing extracellular Ca²⁺ influx, Ca²⁺ release from the internal stores, and Ca²⁺ sensitivity of the myofilaments in pulmonary arteries (14, 15). Furthermore, others have reported that dehydroepiandrosterone treatment prevents both hypoxia-

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and monoketone-induced PH (5, 24, 42). Based on recent findings suggesting NADPH redox and reactive oxygen species modulate protein kinase G (PKG) activity (3, 40), we speculated that increases in Glc-6-PD activity and NADPH levels inhibit PKG in pulmonary arteries, which in turn enhances phenotypic modulation of smooth muscle cells. If so, inhibition of Glc-6-PD could restore the contractile phenotype of smooth muscle cells. Consistent with that idea, we found that inhibition of Glc-6-PD using 6AN or by increasing passive tension reduced persistent HPV by increasing PKG activity and prevented the hypoxia-induced phenotypic modulation of smooth muscle cells.

METHODS

Fourth-order pulmonary artery branches (0.5–1.0 mm inner diameter (id)) were harvested from bovine right and left lungs purchased from a local slaughterhouse (Stuckey’s Meat Packer, Semmes, AL). After quickly excising the heart and lungs from the animal, they were placed in normal Tyrode solution (in mM: 135 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 5 HEPES, and 11 glucose; pH was adjusted to 7.40 with NaOH) and transported to the laboratory on ice. All reagents were purchased from Sigma Chemical (St. Louis, MO).

Western blot analysis. Proteins were extracted from frozen tissue in lysis buffer, after which Western blot analysis using the specific antibodies indicated in the individual figures was performed as described previously (18).

Glucose-6-phosphate dehydrogenase activity. G6PD activity was measured in pulmonary artery homogenates by following the reduction of NADP⁺ to NADPH (18). NADPH fluorescence (excitation, 340 nm; emission, 460 nm) was detected using a microplate reader (Synergy 2 from BioTek Instruments, Winooksi, VT).

Contraction of pulmonary artery. Isolated pulmonary arterial rings, with and without intact endothelium, were prepared from bovine lungs and studied for changes in isometric force as described previously (3). Briefly, arterial rings (0.5–1.0 mm in id and 2 mm in length) were equilibrated in Krebs buffer for 2 h at 1 g passive tension before being contracted with KCl (30 mM) and exposed to treatments as described previously (17, 36). Pulmonary arterial rings were incubated in Krebs solution for 60 min before being contracted with KCl (30 mM) and exposed to hypoxia (95% N₂, 5% CO₂; 20–40 Torr). As a control, the normoxic conditions were 21% O₂, 73% N₂, 5% CO₂, and 140 Torr. The effect of all the vasoreactive drugs used in this study was tested on the basal force, and we found that none of the drugs significantly decrease or increase the baseline contraction of pulmonary arteries.

Immunoprecipitation of PKG. Tissue homogenate was prepared as described above, and PKG was immunoprecipitated by methods described by Dey and Lincoln (9). Self- and co-immunoprecipitation were performed with anti-PKG and anti-Glc-6-PD antibodies, respectively, to determine whether these proteins form a complex. We immunoprecipitated thioredoxin reductase-1 (Trx-R-1) with anti-Trx-R-1 antibody, which is an immunoprecipitation antibody from SantaCruz Biotech, and Western blotting was performed with either anti-PKG or -G6PD antibody.

NADPH levels. Free NADPH was extracted from PA as previously described (28), and levels in PA were determined using a kit from Biovision (Mountain View, CA).

Glutathione levels. GSH was extracted from PA as previously described (28), and levels in PA were determined using a kit from Biovision.

Vasodilator phosphoprotein phosphorylation. Tissue homogenate was subjected to Western blot analysis using anti-phosphorylated vasodilator phosphoprotein (VASP) antibodies (Cell Signaling) to assess PKG-catalyzed phosphorylation of VASP at Ser239 (p-VASP) (7).

Immunofluorescent staining. Frozen sections of pulmonary artery were fixed in acetone, and antigen retrieval was done with 0.1% Triton X-100 as described previously (14, 15, 22). After the sections were blocked by incubation for 1 h in 5% normal goat serum or 5% BSA at room temperature, they were incubated with primary antibody overnight at 4°C. The sections were then washed with 1× PBST and incubated with secondary antibody (anti-mouse Alexa488 or Alexa568 and anti-rabbit Alexa568 or Alexa488, Invitrogen) for 1 h at room temperature. The sections were then washed, stained with DAPI (1 μg/ml), and mounted with DAKO mounting medium. Imaging was done using a Nikon-A1 confocal microscope.

Statistical analysis. Values are presented as means ± SE. ANOVA and post hoc Fisher’s protected t-tests were used for analysis in all studies of vascular contractility. All enzyme activity and Western blot data were analyzed using Student’s t-tests. Values of P < 0.05 were considered significant. In all cases, the number of experimental determinations (n) was equal to the number of lungs from which pulmonary arteries were harvested for this study.

RESULTS

Contraction elicited by persistent hypoxia in isolated pulmonary arteries is endothelium independent. Pulmonary arteries were contracted first with 120 mM KCl and then with 30 mM KCl as shown (Fig. 1A). Arteries precontracted with KCl (30 mM) contracted further upon exposure to hypoxia for 12 h (Fig. 1B). Removing the endothelial layer from the arteries had no effect on the force of contractions elicited by exposure to hypoxia (Fig. 1C). We found that the endothelium-denuded pulmonary arteries contracted when exposed to hypoxia, whereas endothelium-intact pulmonary arteries contracted acutely (within 10–30 min) then relaxed (between 30 and 60 min) and contracted again after 2 h. Thereafter, there was no difference in the maximum active force developed between endothelium-intact and -denuded pulmonary arteries.

Smooth muscle cell phenotype markers and proteins are downregulated in pulmonary arteries exposed to persistent hypoxia. Recent studies have shown that modulation of the smooth muscle phenotype plays a major role in the pathogenesis of vascular diseases (38, 45). Therefore, to determine the phenotype of smooth muscle cells in pulmonary arteries exposed to prolonged hypoxia, cryosections of endothelium-intact pulmonary arteries exposed to hypoxia (12 h) or normoxia were immunostained for two contractile markers, SM-MHC and SM22α, and two cell proliferation markers, PCNA and Ki67 (Fig. 2A). Hematoxylin and eosin staining of pulmonary artery sections (Fig. 2A, first panel shows a ×20 magnification and others show a ×60 magnification) exposed to normoxia or hypoxia revealed no obvious differences in the morphology of the intima, media, or adventitia. Medial staining of SM22α and SM-MHC was observed in endothelium-intact pulmonary artery exposed to both normoxia and hypoxia but was less intense in hypoxic arteries. In addition, PCNA staining was also observed in medial regions under both normoxia and hypoxia, but weak Ki67 staining was present only under hypoxia.

When immunoblotting was performed with protein extracts from isolated pulmonary arteries exposed to hypoxia or normoxia (Fig. 2, B–F), we observed no change in the expression of β-actin and calponin, but SM22α and SM-MHC expression was significantly (P < 0.05) reduced under hypoxia.

Increased Glc-6-PD modulates persistent hypoxia-evoked pulmonary artery contraction and changes in PASM cell phenotype. Because hypoxia-evoked increases in Glc-6-PD levels contribute to acute HPV (15), we investigated the effect of prolonged hypoxia.
hypoxia on Glc-6-PD expression and how this could alter HPV and the phenotype of PASM cells. Exposing pulmonary arteries to hypoxia (12 h) significantly \((P < 0.05)\) increased Glc-6-PD expression (Fig. 3A) and activity (\(>1.5\)-fold), and this effect was attenuated by treating the arteries with a competitive inhibitor, 6AN (1 mM; Fig. 3B). To verify the specificity of 6AN, we examined its effect on the Glc-6-PD activity in lungs from Glc-6-PD deficient mouse model. We found 6AN reduced Glc-6-PD activity in lungs from wild-type (from 1.3 to 0.7 nmol·min\(^{-1}·\text{mg protein}^{-1}\)) but not from Glc-6-PD deficient (from 0.7 to 0.5 nmol·min\(^{-1}·\text{mg protein}^{-1}\)) mice. This is consistent with the earlier observation that 6AN induced relaxation of arteries from wild-type mice but not Glc-6-PD deficient mice (3). To assess the effect of increased Glc-6-PD expression/activity on HPV, we exposed endothelium-intact or -denuded pulmonary arteries to hypoxia or normoxia with or without 6AN. We found that prolonged HPV was significantly blunted by 6AN in the presence and absence of endothelium (Fig. 3, C and D), which suggests Glc-6-PD is involved in mediating HPV.

To determine whether increases in Glc-6-PD activity could influence PASM cell contractile phenotype protein expression, protein extracts from pulmonary arteries treated with 6AN under hypoxia or normoxia were probed for contractile protein/phenotype markers by immunoblotting. 6-AN acts by competing with NADP\(^+\) for the binding site on Glc-6-PD, which it irreversibly blocks (16). Earlier studies, including ours, have shown that 6AN inhibits Glc-6-PD in many cell types and in cancer cells and thus retards cell proliferation and growth (47, 48). For this reason, it is used in cancer therapy as an anti-proliferative/neoplastic agent (16, 25). 6AN treatment had no significant effect on \(\alpha\)- or \(\beta\)-actin expression under normoxia or hypoxia (Fig. 3, E and F). Glc-6-PD inhibition also did not significantly affect SM22\(\alpha\) levels under normoxia, but it increased \((P < 0.05)\) SM22\(\alpha\) levels under hypoxia (Fig. 3G). Similarly, SM-MHC levels were unchanged by 6AN under normoxia but were increased \((P < 0.05)\) under hypoxia (Fig. 3H).

**NADPH levels are increased and GSH are decreased in pulmonary artery by hypoxia.** NADPH and GSH redox is regulated by Glc-6-PD in most cell types. Therefore, we determined whether NADPH and GSH levels are changed in pulmonary artery by acute (10 min) and chronic (12 h) hypoxia. Consistent with our previous findings (15), NADPH levels increased (acutely by 24% and chronically by 98%) and GSH levels decreased (acutely by 15.4% and chronically by 10.3%) in hypoxic compared with normoxic time-matched control pulmonary arteries.

**Inhibition of Glc-6-PD by 6AN activates PKG.** Recent studies, including ours, have shown that inhibition of Glc-6-PD using 6AN activates PKG in coronary and pulmonary arteries (3, 40). We therefore examined whether 6AN-induced inhibition of Glc-6-PD activates PKG in hypoxic pulmonary artery.

We first determined whether PKG regulates Glc-6-PD activity. Inhibition of PKG activity using Rp-\(\beta\)-phenyl-1,\(N^2\)-etheno-8-bromo-guanosine-3',5'-cyclic monophosphorothioate (RT-cGMPs), which inhibits PKG by binding to the cGMP binding site, reduced p-VASP in untreated and KCl-treated pulmonary arteries. This suggests RT-cGMPs inhibited PKG in PASM. RT-cGMPs treatment did not alter Glc-6-PD activity [control 2.0 ± 0.5 and RT-cGMPs (100 nM; 1.8 ± 0.6 nmol·min\(^{-1}·\text{mg protein}^{-1}\)) in the presence of KCl (30 mM)].

As reported previously (15, 19, 20), we observed that Glc-6-PD activity is increased by hypoxia (Fig. 4A). Inhibition of...
PKG did not alter hypoxia-induced increases in Glc-6-PD activity in presence or absence of KCl (Fig. 4A). However, KCl increased p-VASP-to-t-VASP ratios under normoxia, whereas hypoxia reduced (P < 0.05) p-VASP levels (Fig. 4, B and C). RT-cGMPS treatment further decreased p-VASP-to-t-VASP ratios, indicating PKG is inhibited under hypoxic conditions (Fig. 4, B and C).

Glc-6-PD-derived NADPH is an essential co-factor for many reductases in the cell, including TxR, which reduce S-S bonds in proteins (39). Furthermore, Glc-6-PD deficiency or inhibition is known to decrease TxR activity in several mammalian cell types (2). We therefore speculated inhibition of Glc-6-PD activity (under conditions that decrease NADPH) may attenuate TxR activity. Decreased TxR activity could then oxidize cysteine residues in PKG (31) and activate it. If this presumption is correct, then these three proteins should be in close proximity in the cell. Therefore, we performed immunoprecipitation of PKG and TxR-1 from PA homogenates with anti-PKG and -TxR-1 antibody and Western blotting with anti-Glc-6-PD and -PKG antibody. Figure 4, D and E, demonstrates TxR-1, Glc-6-PD, and PKG appear to form a complex in PAs. Intriguingly, Glc-6-PD inhibition by 6AN in the presence of KCl markedly increased p-VASP-to-t-VASP ratios, demonstrating that inhibition of Glc-6-PD activated PKG (Fig. 4, F and G). This led us to implicate that an increase in Glc-6-PD activity inactivates the PKG activity by either up-regulating NADPH under hypoxia and, conversely, that inhibition of Glc-6-PD using 6AN increases PKG activity.

Inhibition of Glc-6-PD by 6AN activates PKG and inhibits hypoxia-evoked decrease of SM22α and pulmonary artery contraction. To assess the role of activated Glc-6-PD in hypoxia-induced contraction and phenotypic modulation of PASM, we
Fig. 3. Glc-6-PD regulation of pulmonary artery smooth muscle cell phenotype. 

A: Glc-6-PD expression was increased in bovine pulmonary arteries exposed to prolonged hypoxia (n = 4). B: 6AN (1 mM; 12 h; n = 5), a potent inhibitor of Glc-6-PD, significantly inhibited Glc-6-PD activity under normoxic and hypoxic conditions in endothelium-denuded pulmonary arteries. C and D: 6AN also reduced hypoxia-induced contractions in endothelium-intact and -denuded pulmonary arteries. E and F: inhibition of Glc-6-PD using 6AN had no effect on α- and β-actin expression in pulmonary arteries exposed to hypoxia or normoxia for 12 h. With Glc-6-PD inhibition under hypoxia, SM22α (G) and SM-MHC (H) expression increased. Normoxic and hypoxic samples were run on one gel in blinded fashion; after the blot was developed, lanes containing samples irrelevant to this figure were removed from the picture.
determined the effect of Glc-6-PD inhibition on the expression of contractile proteins in pulmonary arteries under hypoxia. We found that 6AN upregulated myocardin, the master regulator of several SM contractile protein gene expressions (Fig. 5, A and B). Furthermore, we examined whether 6AN upregulates myocardin expression via PKG pathway. Myocardin expression was increased by 6AN in a PKG-independent manner since RT-cGMPs did not reduce 6AN-evoked increases in myocardin in pulmonary arteries exposed to hypoxia (12 h; Fig. 5, A and B). Consistently, dehydroepiandrosterone, an uncompetitive Glc-6-PD inhibitor, and 6AN also increased myocardin but not SM22α/H9251 expression in PKG-deficient pulmonary artery smooth muscle cells cultured in hypoxia for 72 h (Fig. 5B). We also found that PKG inhibition reduced levels of SM22α and SM-MHC in hypoxic pulmonary arteries and that RT-cGMPs inhibited 6AN-evoked increases in SM22α/SM-MHC/calponin levels without affecting levels of actin (Fig. 5C). Interestingly, PKG inhibition also diminished the 6AN-induced inhibition of hypoxia-induced pulmonary artery contraction (Fig. 5D) without affecting KCl (30 mM)-evoked contraction (control, 94.8 ± 5.4% vs. RT-cGMPs, 98.4 ± 7.2% of 120 mM KCl).

Stretch stimulates Glc-6-PD- and PKG-dependent phenotypic modulation in pulmonary arteries exposed to hypoxia. Pulmonary arteries are highly compliant, but the increases in pulmonary artery pressure seen under hypoxia puts a high degree of stress on the vessel walls (44). We therefore studied the effect of increasing passive tension on the pulmonary artery wall on expression of Glc-6-PD and PASM phenotypic markers. Pulmonary arterial levels of calponin and ß-actin were unaffected by increasing passive tension (from 0.8 to 3 g) in hypoxia or normoxia (representative arteries from three different animals were pooled together to obtain sufficient protein for immunoblotting). However, surprisingly, we found that levels of Glc-6-PD decreased, whereas both p-VASP and...
SM22α increased with increases in passive tension under hypoxia (Fig. 6A). When KCl-induced contractions were elicited in arteries at different levels of passive tension, we found that chronic hypoxia (12 h) increased the force of contraction at low (0.8 g) passive tension but led to relaxation upon application of higher (3.0 g) passive tension (Fig. 6B). We speculated that the relaxation seen under hypoxia with higher tension could partly be due to upregulation of PKG activity under hypoxia, which may lead to less actin–myosin crossbridge cycling and loss of contractile force. To assess actin–myosin interactions, we immunostained frozen sections of pulmonary arterial rings subjected to 0.8 or 3.0 g of tension under normoxia and hypoxia. We observed increases in the interaction (orange to yellow) of actin (green) with SM-MHC and SM-pMLC (both red) under chronic hypoxia at 0.8-g tension. With 3.0 g of tension, by contrast, we saw no increase in the α-actin-SM-MHC or α-actin-SM-pMLC interaction (Fig. 6C). Furthermore, co-immunoprecipitation of α-actin and SM-MHC (Fig. 6D) confirms the immunofluorescence findings.

**DISCUSSION**

In the present study, we have shown that exposing the pulmonary artery to hypoxia increases active contraction and decreases expression of the contractile proteins SM22α and SM-MHC. Interestingly, Glc-6-PD inhibition led to activation of PKG, which in turn inhibited the hypoxia-induced phenotypic modulation in PASMC cells and attenuated hypoxia-induced contraction of PAs.

Cells and organisms are able to very quickly adapt biochemically and physiologically to changes in PO2 tension (21). It has been known for >100 yr that systemic arteries dilate and pulmonary arteries constrict in response to hypoxia, and numerous studies have been conducted to decipher the molecular mechanisms involved in this adaptation (1, 21, 29). Although identity of the PO2 sensor is somewhat known, the effectors that mediate hypoxic responses within blood vessels still remains unknown. The PO2 sensor rapidly detects changes in PO2, and vascular smooth muscle quickly (within minutes) responds by either contracting or relaxing (21).

Pulmonary arteries contract in response to hypoxia to divert blood away from poorly ventilated areas toward well ventilated areas of the lungs. In addition, hypoxia stimulates the synthesis and release of endothelium-derived factors (endothelin, prostanoi, thromboxanes, and serotonin) that all contribute to the hypoxic contraction (29, 49). Our results suggest that endothelium influences the contractility of pulmonary arteries in the early phase of the HPV response.

A number of theories have been put forth to explain how hypoxia increases active force: inhibition of Kv1.5 channel function and expression, activation of Ca2+/store-operated Ca2+ channels, potentiation of Ca2+ release from internal stores, and increased sensitivity of the myofilaments to Ca2+ are all thought to contribute to HPV (29, 49). Our earlier studies showed that Glc-6-PD mediates acute HPV and that hypoxia does not evoke contraction in pulmonary arteries isolated from Glc-6-PD-deficient mice. Concur-
Currently, other studies showed that Glc-6-PD inhibitors reduce PH (5, 15, 19, 20, 24, 42). We now found that stimulation of PKG (measured as VASP phosphorylation) through Glc-6-PD inhibition reduces hypoxia-induced pulmonary artery contraction, and this inhibition is partially reversed by RT-cGMPs, a PKG inhibitor. Our results also suggest that TxR-1, Glc-6-PD, and PKG form a complex. We therefore propose that PKG activation, presumably via redox changes (3, 40), secondary to Glc-6-PD and TxR-1 inhibition is another novel pathway via which Glc-6-PD inhibitors reduce HPV.

We also found that hypoxia induces a reduction in pulmonary arterial expression of SM22α and SM-MHC expression without affecting actin expression. Consistent with those results, hypoxia is known to reduce the promoter activity of multiple smooth muscle cell marker genes in ovine fetal pulmonary venous smooth muscle cells and in primary PASM cells (26, 52) and to facilitate transdifferentiation of endothelial and progenitor cells into PASM cells (27, 53). It has been suggested that downregulation of SM22α and tropomyosin expression reduces hypoxia-induced relaxation of porcine coronary arteries (46), but the physiological function of SM22α in smooth muscle is only poorly understood (23, 37). For example, it remains controversial whether SM22α binds to actin (12, 37). Some studies suggest active force is reduced in mesenteric resistance arteries and portal veins from SM22α−/− mice (51), whereas others have proposed that SM22α inhibits actin-myosin interaction (46). Moreover, generally, changes in SM22α expression are associated with the phenotypic modulation of smooth muscle (37, 43). Likewise, reduced expression of SM-MHC is also thought to reflect phenotypic modulation of smooth muscle (10). Consistent with that notion, we also found that cell cycle/proliferative proteins (PCNA and Ki-67) slightly increased in hypoxic PASM. Our results thus suggest hypoxia induces arterial contraction along with biochemical changes that foster PASM proliferation and contribute to the remodeling of the pulmonary artery to protect against the higher (from 20 to 21) pulmonary arterial pressure.

Fig. 6. The effect of increasing passive tension on hypoxia-induced pulmonary artery contraction and changes in smooth muscle phenotype marker levels. A: increasing passive tension had no effect on expression of calponin or β-actin in pulmonary arteries exposed to prolonged hypoxia or normoxia (representative of arteries from three different animals were pooled together to obtain sufficient protein for immunoblotting). However, SM22α and p-VASP expression increased with increasing passive tension under hypoxia but not normoxia. B: prolonged hypoxia (12 h) increased arterial contraction in the presence of low (0.8 g) passive tension, but application of 3 g of tension led to relaxation. C: immunofluorescent staining of frozen sections of pulmonary arterial rings subjected to 0.8 or 3 g of passive tension under normoxia or hypoxia shows increased interaction (orange to yellow) of α-actin (green) with SM-MHC or SM-pMLC (both red) under prolonged hypoxia with 0.8 g of passive tension (representative of two micrographs), but there is no change in α-actin-SM-MHC or α-actin-SM-pMLC interaction with 3 g of passive tension. D: co-immunoprecipitation of α-actin-SM-MHC is decreased by 6AN (1 mM) treatment.
Changes in the expression of contractile proteins induced by persistent hypoxia were also prevented by 6AN, which acts by competitively inhibiting Glc-6-PD (16). Interestingly, treating pulmonary artery with 6AN for 12 h increased expression of SM22α and SM-MHC. This suggests that 6AN prevented or reduced HPV by simultaneously reducing Ca²⁺-mediated active force development (15) and inhibiting the phenotypic modulation of smooth muscle cells (Fig. 3). This response appears to be mediated via the PKG pathway, since the effects of 6AN, including increased phosphorylation of VASP, was inhibited by RT-cGMPs. These results clearly demonstrate that hypoxia induces activation of Glc-6-PD and reduces levels of p-VASP and that hypoxia-induced modulation of PASM cell phenotype was mediated by Glc-6-PD via the PKG pathway.

PKG is well known to control vascular smooth muscle phenotype, in part by stimulating myocardin/serum response factor (SRF)-dependent gene expression (8, 33). Inhibition of PKG in ovine fetal pulmonary venous smooth muscle cells downregulates the expression of myocardin but upregulates expression of Elk-1 (52). Alternatively, PKG may reduce Elk-1 activity through sumo modification, thereby increasing myocardin-SRF-dependent smooth muscle-specific gene expression in vascular smooth muscle cells (8). Because downregulation (by 52.14%) of myocardin expression in hypoxic pulmonary arteries was prevented by 6AN via a PKG-independent pathway, we suggest that activation of PKG by 6AN reduces Elk-1-induced inhibition of SRF-sensitive smooth muscle-specific genes, like SM22α.

Expression of PKG in PKG-deficient vascular smooth muscle cell line results in an increased production of contractile phenotype marker proteins such as SM-MHC, calponin, and α-actin (4). Additionally, myoendothelial gap-junctional signaling also plays a role in upregulating the expression of these contractile proteins in pulmonary artery smooth muscle cells co-cultured with endothelial cells (13). It is, therefore, reasonable to speculate that endothelium-derived signals may influence the expression of SM-MHC and SM22α proteins in intact pulmonary artery as well. Since hypoxia diminished PKG activity in pulmonary artery, we speculate that this might have resulted in decreasing SM22α and SM-MHC expression in pulmonary artery.

Moreover, as illustrated in a schematic (Fig. 7), our results provide novel evidence for additional Glc-6-PD-dependent redox- and O2 tension-sensitive mechanisms that inactivate PKG and control the expression of these contractile proteins in smooth muscle.

The tensile stress added to the vascular wall by increasing pressure is a physical/mechanical factor that stimulates pulmonary arterial remodeling in PH (34). Increases in pulmonary arterial pressure have been shown to depolarize PASM cell membranes in pulmonary hypertensive rats (6, 50). Wall stress-induced deformation of elastic laminae and thickening of extracellular matrix is also thought to evoke pulmonary artery remodeling in chronic hypoxia-induced PH (30, 34). Moreover, static stretch is known to promote DNA synthesis in cultured rat pulmonary arteries (50) and increase KCl-induced active contraction of bovine pulmonary arteries (41) but reduce active circumferential tension and stress in vessels from monocrotaline-treated rats (32). Although physical stress had no obvious effect on the expression of vascular smooth muscle phenotype markers under normoxia, their expression was altered by increases in passive tension in hypoxic pulmonary arteries. Intriguingly, Glc-6-PD expression declined linearly with increasing passive tension under hypoxia, and expression of both p-VASP and SM22α was increased. We also observed that arteries resting at 3.0 g of passive tension relaxed instead of contracted in response to hypoxic stimuli. We suggest that a reduction in actin-myosin interaction evoked by the increase in PKG activity could be responsible for this phenomenon. Our novel results suggest that, in pulmonary arteries, Glc-6-PD contributes to hypoxia- and stress-induced decreases in the PKG-dependent phosphorylation of VASP as well as the expression of cytoskeletal/phenotype proteins.

PH is a debilitating multifactorial illness with no effective treatment (11). Persistent reductions in inhaled oxygen or generalized hypoxia is a cause of secondary PH in COPD (bronchitis, emphysema, asthma, and lung cancer) patients and individuals living at high altitude (45). In idiopathic or familial primary pulmonary arterial hypertension, constriction/obliteration of the pulmonary arteries causes hypoxia (38, 45). However, the molecular mechanism(s) by which hypoxia evokes pulmonary artery constriction and remodeling during the pathogenesis of PH is still not well understood (11). We found that hypoxia stimulates Glc-6-PD, which increases pulmonary artery contraction, and speculated that activated Glc-6-PD

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**Fig. 7.** Glc-6-PD and thioredoxin reductase-1 (TxR-1) form a complex with PKG and regulate hypoxia-evoked changes in the expression of contractile proteins in pulmonary artery. Schematic illustrating a potential pathway through which hypoxia affects activity of Glc-6-PD and PKG and expression of contractile proteins. Inhibition of Glc-6-PD-derived NADPH redox reduces TxR-1 activity, which oxidizes thiols on PKG and activates it. Increase in PKG activity reexpresses contractile proteins, whereas myocardin expression is increased in a PKG-independent manner.
contributes to persistent HPV and PH. The present findings indicate that increases in Glc-6-PD activity influence contraction, PKG activity, and PASM cell phenotype, all of which affect pulmonary artery remodeling. We believe that a fuller understanding of how Glc-6-PD regulates pulmonary arterial phenotype and vasomotor tone could lead to the development of new strategies to reverse PH.

In summary, we propose that, within PASM cells, Glc-6-PD serves as a link between the PO2 sensor and the effectors that mediate HPV. If so, Glc-6-PD could be an important new therapeutic target. Indeed, our findings suggest that inhibition of Glc-6-PD could simultaneously diminish activity in several pathways that promote PH.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


