

# Hypoxic Regulation of Pulmonary Vascular Smooth Muscle Cyclic Guanosine Monophosphate–Dependent Kinase by the Ubiquitin Conjugating System

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We previously reported that hypoxia attenuates nitric oxide–cyclic guanosine monophosphate (NO–cGMP)–mediated fetal pulmonary vessel relaxation by inhibiting cGMP-dependent protein kinase 1 (PKG1) activity, but not all the mechanisms by which acute hypoxia inhibits PKG1 activity have been delineated. Here we demonstrate for the first time, to the best of our knowledge, that acute hypoxia induces an accumulation of ubiquitinated PKG1 in ovine fetal and newborn pulmonary artery smooth muscle cells. Such a modification was not evident in ovine fetal systemic (cerebral) artery smooth muscle cells. The accumulation of polyubiquitinated PKG1 observed after 4 hours of hypoxia was affected neither by the activation of PKG1 kinase activity with the cell-permeable cGMP analogue 8-bromo-cGMP, nor by its inhibition with DT-3 in fetal pulmonary artery smooth muscle cells. Ubiquitinated PKG1 $\alpha$  was unable to bind the cGMP analogue 8-(2-aminoethyl)thioguanosine-3',5' (AET)-cGMP, a ligand for the unmodified protein. Inhibition of the proteasomal complex with MG132 led to the accumulation of polyubiquitinated PKG1 in normoxia, indicating the involvement of the ubiquitin-26S proteasomal system in degradation and clearance of this protein under normoxic conditions. The ubiquitinated PKG1 under hypoxic conditions, however, was not predominantly targeted for proteasomal degradation. Importantly, reoxygenation reversed the acute hypoxia-induced accumulation of ubiquitinated PKG1. Our results suggest that the PKG1 ubiquitination induced by acute hypoxia plays a unique role in the regulation of the pulmonary vascular smooth muscle cell vasoreactivity and relaxation mediated by the NO–cGMP–PKG1 pathway.

**Keywords:** cGMP-dependent protein kinase; proteasome; ubiquitin conjugation; vascular smooth muscle; hypoxia

Cyclic guanosine monophosphate (cGMP)–dependent protein kinase (PKG1) is a serine–threonine kinase that plays an important role in nitric oxide–cyclic guanosine monophosphate (NO–cGMP)–mediated relaxation (1). In the canonical pathway, endothelium-derived NO diffuses into the vascular smooth muscle cell (VSMC) layers and activates soluble guanylate cyclase (sGC) to elevate concentrations of cyclic guanosine 3',5'-monophosphate (cGMP). cGMP activates PKG1 to phosphorylate a wide range of downstream targets involved in the regulation of actomyosin cytoskeleton, calcium-mediated signaling

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## CLINICAL RELEVANCE

Understanding the control of vasomotor tone in the fetal and neonatal circulations is critical to the treatment and prevention of pulmonary vascular disorders such as persistent pulmonary hypertension of the newborn. Our study implicates a new ubiquitin-mediated pathway in the regulation of nitric oxide–cyclic guanosine monophosphate–dependent protein kinase function in hypoxia, providing additional insights into the mechanisms controlling vasorelaxation, and potentially elucidating some mechanisms of hypoxic pulmonary vasoconstriction.

mechanisms, and K<sup>+</sup> channel function, resulting in vasodilatation (reviewed in detail elsewhere) (2, 3). The N-terminus of PKG1 (~ 100 amino acids) is encoded by two alternative exons, resulting in the expression of two isoforms, PKG1 $\alpha$  and PKG1 $\beta$  (apparent molecular weights of ~ 77 and ~ 80 kD, respectively) in an apparently spatiotemporal manner (4). Together, the PKGs regulate the smooth muscle cell “contractile state,” phenotype, and proliferative behavior (5–7).

Acute hypoxia is a known physiological stimulus for pulmonary vasoconstriction and increased pulmonary vascular resistance, unlike the systemic circulation. We showed previously that both fetal and neonatal ovine pulmonary vessels exhibit a vigorous dilator response to the nitric oxide–cGMP pathway, and that PKG1 plays a prominent role (8, 9). We also showed that acute hypoxia attenuates PKG1 activity in fetal and newborn pulmonary vessels and vascular smooth muscle cells, and that one mechanism by which this occurs involves reactive species–mediated PKG1 nitration and inactivation (10–12). Other mechanisms may be involved in the regulation of PKG1 activity in hypoxia. Sustained hypoxia-induced decreases in concentrations of PKG1 are associated with changes in smooth muscle contractile function and phenotypic modulation (7, 13, 14). However, the actual mechanisms involved in the regulation of PKG1 activity in hypoxia are unclear. The ubiquitin proteasome pathway is a major intracellular proteolytic system in eukaryotes, responsible for the protein homeostasis that also plays a critical role in the cellular hypoxic response (15–17). Here, we tested the hypothesis that acute hypoxia modulates PKG1 activity by posttranslational modification via an ubiquitin-dependent pathway in pulmonary vascular smooth muscle cells.

## MATERIALS AND METHODS

### Fetal and Newborn Lamb Pulmonary Artery Smooth Muscle Cell Culture

All animal procedures and protocols described were reviewed and approved by the Animal Care and Use Committee at the University of

Illinois. Fetal pulmonary artery smooth muscle cells (FPASMCs) and newborn pulmonary artery smooth muscle cells were isolated from intrapulmonary arteries (fourth to sixth generation vessels; see the online supplement) of near-term ovine fetuses and 1–2-day-old newborn lambs, using methods previously described (18), and cultured in Dulbecco's Modified Eagle's Medium containing 10% FBS. Cells were immunostained for  $\alpha$ -smooth muscle actin (monoclonal antibody; Sigma-Aldrich, St. Louis, MO) to ascertain the smooth muscle phenotype (greater than 98%), and used between passages 3–6. Similarly, systemic vascular smooth muscle cells were isolated from fetal cerebral arteries (FCASMCs).

### Exposure of Cells to Acute Hypoxia

Cells were starved for 3 hours in medium containing 0.2% FBS before hypoxia treatment (unless mentioned otherwise) in a humidified incubator saturated with 5% CO<sub>2</sub>, 3% oxygen, and balance nitrogen (Forma 3130; Thermo Scientific, Asheville, NC). The medium contained 2% FBS in experiments involving greater than 4 hours of exposure to hypoxia. The medium was rendered hypoxic by pre-equilibrating it in the hypoxia incubator for 3 hours before use. Normoxic control samples were placed in a standard CO<sub>2</sub> incubator with 21% oxygen (Heracell240; Thermo Scientific). The PO<sub>2</sub> in the medium exposed to hypoxia or normoxia measured between 30–40 mm Hg and approximately 100 mm Hg, respectively, and was monitored using a dissolved oxygen meter (Model 407510; Extech Instruments, Waltham, MA). Reoxygenation experiments involving hypoxia-exposed cells were performed by exposing FPASMCs to 2 hours of hypoxia (3% O<sub>2</sub>), after which the hypoxic medium was replaced with normoxic medium (21% O<sub>2</sub>), and the cells were returned to the normoxic incubator for 2 hours.

### Transfection, Immunoprecipitation, and Western Blot Analysis of PKG1 and Ubiquitinated PKG1

Transient transfection with a plasmid encoding myc-tagged, full-length PKG1 $\alpha$  was performed using Lipofectamine (Invitrogen, Carlsbad, CA). Unless otherwise mentioned, cell extracts were prepared in cold RIPA (radioimmunoprecipitation assay) cell lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100) containing a cocktail of protease inhibitors. Protein content was determined using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Cell extracts containing same amount of protein were mixed with 2  $\mu$ g of PKG1 (rabbit polyclonal; Assay Designs, Ann Arbor, MI) or the PKG1 $\alpha$  (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) antibody for immunoprecipitation. For Western blot analysis, the proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), blocked with 5% nonfat milk in TBS-T (Tris-buffered saline-Tween; 20 mM Tris-Cl, 137 mM NaCl, and 0.05% Tween-20, pH 8.0), and incubated with anti-ubiquitin antibody (monoclonal; Assay Designs). After incubation with horseradish peroxidase-conjugated secondary antibody, the blots were developed by the Supersignal West Pico Chemiluminescent Substrate (Pierce). Cell extracts were also probed with PKG1 (rabbit polyclonal; Assay Designs) or actin (rabbit polyclonal; Santa Cruz Biotechnology) antibody. When necessary, the blots were stripped (Restore PLUS; Pierce) for reprobing with the indicated antibodies. Blots were scanned and band intensities were quantified using the free software ImageJ (National Institutes of Health, Bethesda, MD).

## RESULTS

### Acute Hypoxic Exposure Leads to Ubiquitin Modification and Accumulation of Ubiquitinated PKG1 in Ovine FPASMCs

To determine whether hypoxia modifies PKG1 post-translationally by ubiquitin modification, resulting in decreased PKG1 activity, we analyzed ovine FPASMCs that had been exposed to acute hypoxia or normoxia in the presence or absence of MG132, a specific cell-permeable proteasome inhibitor. Ubiquitin conjugation was confirmed by immunoblot analysis with anti-ubiquitin (anti-Ub) antibody after the immunoprecipitation of PKG1 species, using a common anti-PKG1 antibody that recognizes both PKG1 $\alpha$  and PKG1 $\beta$ . The inhibition of proteasomes with MG132 resulted in a noticeable accumulation of polyubiquitinated PKG1 (Ub-PKG) in normoxic cells (Figure 1A;

compare *lane 2* with *lane 1*), suggesting that PKG1 is degraded by the ubiquitin-26S proteasome system (UPS). Unexpectedly, the exposure of cells to hypoxia *per se* resulted in a drastic accumulation of Ub-PKG (Figure 1A; compare *lane 3* with *lane 1*). Some additional accumulation of Ub-PKG species was evident after the inhibition of proteasomes with MG132 (Figure 1A; *lane 4* versus *lane 3*), indicating that UPS degrades only a portion of Ub-PKG in hypoxia. Notably, hypoxia did not enhance the total concentration of ubiquitinated proteins in FPASMC lysates (Figure 1B; *lane 3* versus *lane 1*), and the inhibition of proteasomes with MG132 resulted in an accumulation of polyubiquitinated proteins in both normoxic and hypoxic cells (Figure 1B; compare *lane 2* with *lane 1*, and *lane 4* with *lane 3*). In addition, exogenous PKG1 $\alpha$  was also modified by ubiquitin during acute hypoxia in pulmonary artery smooth muscle cells (Figure 1C; compare *lane 3* with *lane 2*). The modification by ubiquitin of PKG1 was not dependent on the serum concentration in the experimental culture medium, because the phenomenon was observed in low serum-containing (0.2% FBS) and high serum-containing (2–5% FBS) medium. Thus, acute hypoxia appears to activate PKG1-specific E2-ubiquitin-conjugating and/or E3-ubiquitin-conjugating enzymes in FPASMCs, and a significant proportion of Ub-PKG in hypoxia is not targeted for proteasomal degradation.

### Ubiquitination of PKG1 in Hypoxia Is Specific to Pulmonary Arterial Smooth Muscle Cells

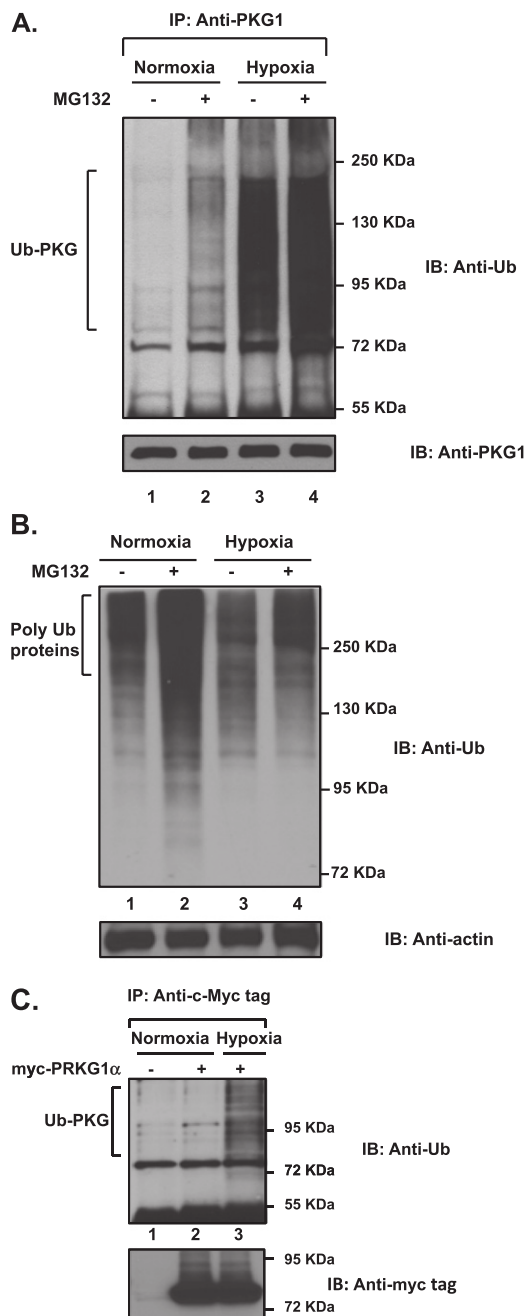
To determine the specificity of acute hypoxia-induced PKG1 ubiquitination in pulmonary VSMCs versus systemic VSMCs, isolated ovine FCASMCs were studied in the same manner as already described, in the presence or absence of the proteasomal inhibitor MG132 (Figure 2). No Ub-PKG was observed under normoxic conditions in any smooth muscle cells (Figure 2A, *top*; *lanes 1* and *5*). MG132 treatment under normoxic conditions resulted in an accumulation of Ub-PKG in both cell types (Figure 2A, *top*; compare *lane 2* with *lane 1*, and *lane 6* with *lane 5*), suggesting that UPS regulates concentrations of PKG1, and the ubiquitinated PKG1 is rapidly degraded. The amount of Ub-PKG in cerebral artery smooth muscle cells (SMCs) after the inhibition of proteasomes was significantly greater compared with that in pulmonary artery SMCs (Figure 2A, *top*; compare *lane 6* with *lane 2*), suggesting a shorter half-life of PKG1 in these cells. We noticed that cerebral SMCs contain substantially lower concentrations of endogenous PKG1, compared with FPASMCs (Figure 2B), consistent with its increased degradation rate via the UPS. Remarkably, hypoxic exposure *per se* induced the accumulation of Ub-PKG species only in pulmonary SMCs (Figure 2A; compare *lane 3* with *lane 7*). Notably, the stripped blots reprobbed with PKG1 antibody also showed polyubiquitinated PKG1 species (Figure 2A, *bottom*).

### Fetal and Newborn Pulmonary Arteries Do Not Demonstrate Increased Ubiquitinated PKG1

Fetal arteries, when dissected in a hypoxic chamber so that they remain chronically hypoxic as *in utero*, did not contain an increased amount of ubiquitinated PKG1. Neither did newborn pulmonary arteries that were normoxic (Figures 3A and 3B). However, the modification by ubiquitin of PKG1 was increased in both fetal and newborn pulmonary artery SMCs upon exposure to acute hypoxia (Figure 3C).

### Acute Hypoxia Induces Transient Accumulation of Ubiquitinated PKG1 in Ovine FPASMCs

To characterize the observed phenomenon further, FPASMCs were exposed to hypoxia for different time intervals. We found



that Ub-PKG rapidly accumulated until 4 hours of hypoxic exposure, and thereafter the amount of polyubiquitinated PKG1 species gradually decreased to an undetectable level by 16 hours of hypoxic treatment (prolonged) (Figure 4A, *top*). An immunoblot analysis of cellular lysates revealed that the concentration of PKG1 was markedly reduced after 16 hours of hypoxic exposure (Figure 4B; by  $\sim 30\%$  and  $\sim 40\%$  for total PKG1 and PKG1 $\alpha$ , respectively). No accumulation of polyubiquitinated PKG1 species was evident in cells maintained in normoxia during this same time period (data not shown).

#### PKG1 Ubiquitination in Acute Hypoxia Is Reversible upon Reoxygenation

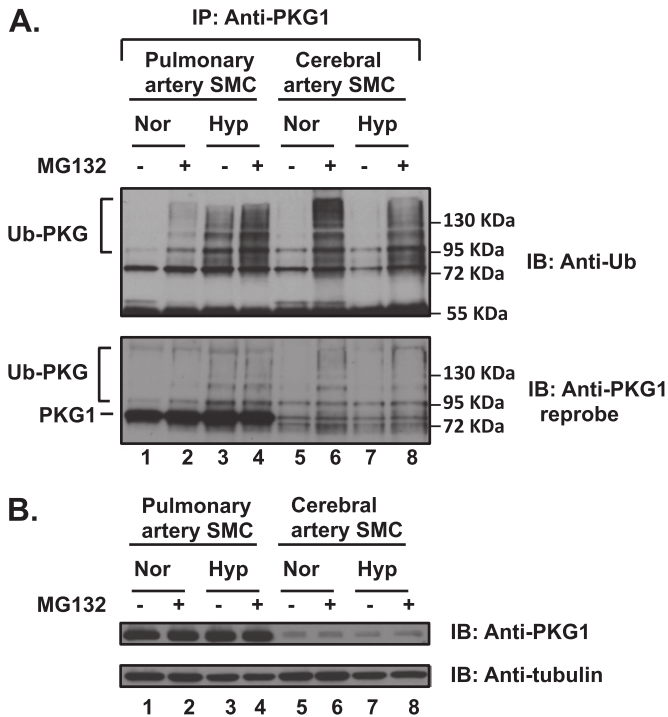
An analysis of cell extracts revealed that PKG1 was progressively ubiquitin-conjugated during the 2 hours of hypoxic

exposure (Figure 5, *top*; lanes 2 and 3), but after 2 hours of reoxygenation of the cells, polyubiquitinated PKG1 was effectively cleared (Figure 5; lane 4). In these experiments, the protein concentrations of PKG1 were not decreased significantly after 4 hours of hypoxia. The disappearance of the polyubiquitinated PKG1 species after reoxygenation suggests that the ubiquitinated protein was either rapidly degraded or de-ubiquitinated upon reoxygenation. Thus, the hypoxically induced accumulation of Ub-PKG is temporary and reversible.

#### PKG1 Ubiquitination in Hypoxia Is Not Affected by PKG Kinase Activity, and Is Not Mediated by Reactive Oxygen Species

The relationship of endogenous intracellular PKG1 serine-threonine kinase activity to ubiquitination was evaluated in acute hypoxia. Neither a 2-hour treatment of FPASMCs with 0.5 mM 8-bromo-cGMP (the phosphodiesterase-resistant analogue of cGMP, used to stimulate PKG activity) nor an inhibition of constitutive PKG1 kinase activity with 5  $\mu\text{M}$  of the cell-permeable peptide DT-3 changed the level of PKG1 ubiquitin modification during acute hypoxia (Figure 6 A, lanes 5 and 6 versus lane 4). Neither of the compounds induced the ubiquitination of PKG1 under normoxic conditions (Figure 6 A, lanes 2 and 3 versus lane 1). This indicates that the hypoxically induced accumulation of Ub-PKG is not dependent on constitutive cellular PKG1 kinase activity.

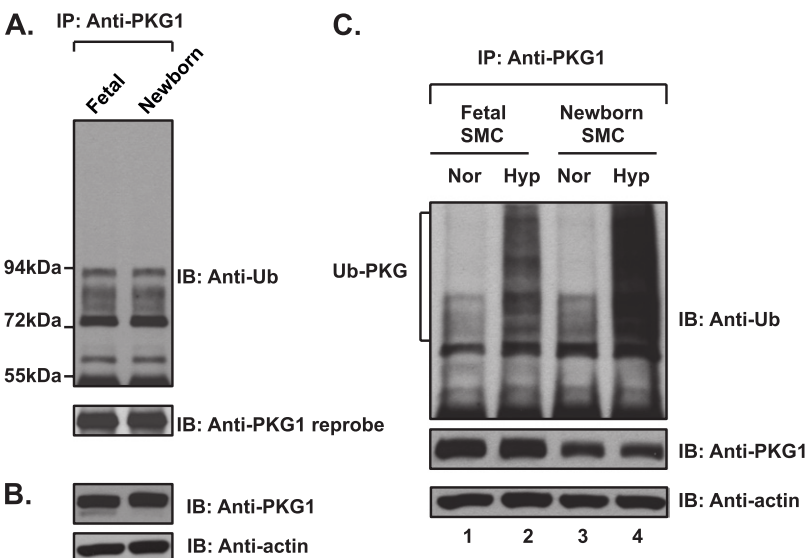
To address whether the reactive oxygen species (ROS) and nitrogen species generated in hypoxia are involved in the ubiquitination of PKG1, FPASMCs were exposed to acute hypoxia in the absence or presence of various scavengers of reactive species, namely, trolox (a vitamin E-derivative and selective peroxynitrite scavenger), quercetin (an antioxidant), and *N*-acetylcysteine (a general ROS scavenger) (Figure 6B). In every case, treatment with the ROS inhibitor/scavenger did not affect PKG1 ubiquitination under hypoxic conditions. Thus, the reactive species generated in hypoxia may not be involved in acute hypoxia-induced PKG1 ubiquitination.



**Figure 2.** Hypoxia-induced ubiquitination of PKG1 is specific to pulmonary artery smooth muscle cells (SMCs). Isolated FPASMCs and fetal cerebral artery smooth muscle cells (FCASMCs) were both exposed to hypoxia (Hyo) or normoxia (Nor) in the presence or absence of the proteasomal inhibitor MG132 (5 μM) for 4 hours. (A) The cell lysates were immunoprecipitated with the common PKG1 antibody and assessed using Western blotting with anti-ubiquitin monoclonal antibody, as described in MATERIALS AND METHODS. Blots were stripped and reprobbed with anti-PKG1 antibody. (B) The cell lysates were probed with a common PKG1 antibody, and tubulin served as the loading control. Experiments were repeated 3–4 times, and a representative blot is shown. The ubiquitination of PKG (Ub-PKG accumulation) was evident in hypoxia only in FPASMCs, and not in FCASMCs.

**Ubiquitinated PKG Exhibits Altered Ligand Binding Affinity**

To determine whether hypoxically induced PKG1 ubiquitination may alter some functional activity of the protein, we tested its



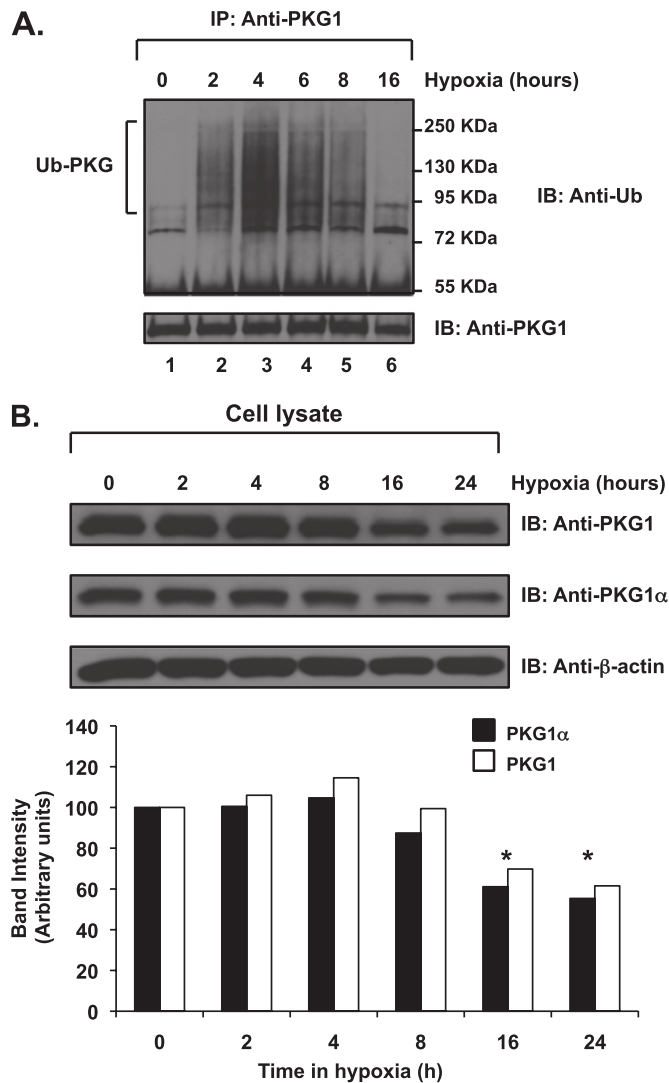
**Figure 3.** Fetal and newborn pulmonary arteries do not demonstrate increased ubiquitin modification of PKG1. The ubiquitin modification of PKG1 is increased in both fetal and newborn pulmonary artery SMCs upon exposure to acute hypoxia. (A) Fetal arteries were isolated under hypoxic conditions as *in utero*. Ovine fetal and newborn pulmonary artery extracts were immunoprecipitated with anti-PKG1 antibody and analyzed by Western blotting, using an anti-ubiquitin antibody. The blot was reprobbed with anti-PKG1 antibody. (B) The arterial tissue extracts were analyzed by Western blotting, using anti-PKG1 and actin antibodies. (C) Fetal and newborn ovine pulmonary artery SMCs were exposed to hypoxia or normoxia for 3 hours. Cell lysates were immunoprecipitated with PKG1 antibody and assessed by Western blotting, using an anti-ubiquitin monoclonal antibody (*top*). Blots were reprobbed with PKG1 antibody (*middle*). Actin in cell extracts served as loading control (*bottom*). A representative blot from three experiments is shown.

ability to bind the cGMP analogue. We performed a large-scale purification of PKG1 species from hypoxic and normoxic FPASMC extracts, using 8-(2-aminoethyl)thioguanosine-3',5' (AET)-cGMP coupled to agarose-bead affinity chromatography. Polyubiquitinated PKG1α rapidly accumulated within the cells after 3 hours of hypoxic exposure (Figure 7A, *top*; lane 2 versus lane 1). Analysis of the 8-AET-cGMP bound and unbound protein fractions revealed that the hypoxia-induced polyubiquitinated PKG1α remained entirely in the unbound fraction (Figure 7A, *top*; lane 6 versus lane 4). PKG1α, unmodified by ubiquitin conjugation, was efficiently bound and eluted from the 8-AET-cGMP ligand beads by cGMP from either normoxic or hypoxic lysates (Figure 7A, *bottom*, lanes 3 and 4), with only a minor amount parting with the unbound fraction (Figure 7A, *bottom*, lanes 5 and 6). No significant difference was evident between the amount of bead-bound unmodified PKG1α in hypoxic and normoxic samples (Figure 7A, *bottom*; compare lane 4 with lane 3). Actin loading control and PKG1 protein levels were similar in the cell extracts. This result suggests that polyubiquitinated PKG1α is unable to bind the cGMP analogue 8-AET-cGMP.

**DISCUSSION**

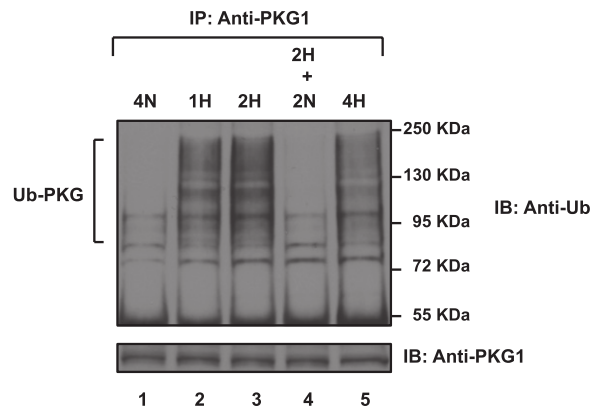
Here we describe a novel regulatory mechanism wherein acute hypoxia leads to the accumulation of Ub-PKG with altered ligand affinity, and protracted hypoxia likely inhibits the NO-cGMP-PKG vasodilatory pathway by engendering the degradation of polyubiquitinated PKG1. Acute hypoxia posttranslationally modifies PKG1 by ubiquitin conjugation in ovine fetal and newborn pulmonary artery SMCs (Figures 1, 2, and 4). Because the UPS is the major nonlysosomal protein-degradation pathway in eukaryotic cells, we surmised that PKG1 is also a target for this system during both normoxia and hypoxia. FPASMCs, after treatment with proteasomal inhibitors under normoxic conditions, accumulated polyubiquitinated PKG1 protein (Figures 1 and 2). The accumulation of polyubiquitinated PKG1 observed during acute hypoxia was further enhanced in the presence of the MG132 compound (Figures 1 and 2). This indicates that PKG1 is also a target for UPS-mediated degradation during acute hypoxia.

Hypoxically induced PKG1 ubiquitin conjugation was not evident when cells were reoxygenated after hypoxic exposure (Figure 5). This parallels our earlier observations that the nitration of PKG1 during hypoxia is not evident after reoxygenation, and



**Figure 4.** PKG1 is subject to time-dependant ubiquitination in acute hypoxia. Ovine fetal pulmonary vascular smooth muscle cells were exposed to normoxia or hypoxia. (A) Cell extracts were immunoprecipitated with a common PKG1 antibody and analyzed by SDS-PAGE and Western blotting, using an anti-ubiquitin monoclonal antibody. The blots were stripped and reprobed with PKG1 antibody. (B) Cell lysates were analyzed by Western blotting, using a common PKG1 antibody or PKG1 $\alpha$  isoform-specific antibody. Gel loading controls were assessed by probing with an actin antibody. *Bottom:* A plot of the relative PKG1 protein band intensities, normalized to actin levels, was quantified using ImageJ software (National Institutes of Health), and expressed as a percentage. Experiments were repeated 4–5 times, and representative blots are shown. \* $P < 0.05$ . The data shown are for hypoxia experiments. No PKG1 ubiquitination during normoxia was observed (data not shown).

that the recovery of PKG activity occurs upon reoxygenation. Our earlier experiments related to the nitration of PKG1 were performed from 30 minutes to 4 hours of exposure to hypoxia in pulmonary venous smooth muscle cells (11). The interrelationship between PKG1 nitration and PKG1 ubiquitination in hypoxia, if any, remains to be determined. The accumulation of polyubiquitinated PKG1 induced by hypoxia is not affected by the endogenous activation of PKG serine-threonine kinase activity by a cGMP analogue, or by the endogenous inhibition of PKG1 activity via a cell-permeable inhibitor (Figure 6A). This hypoxically induced acute effect on PKG1 in pulmonary

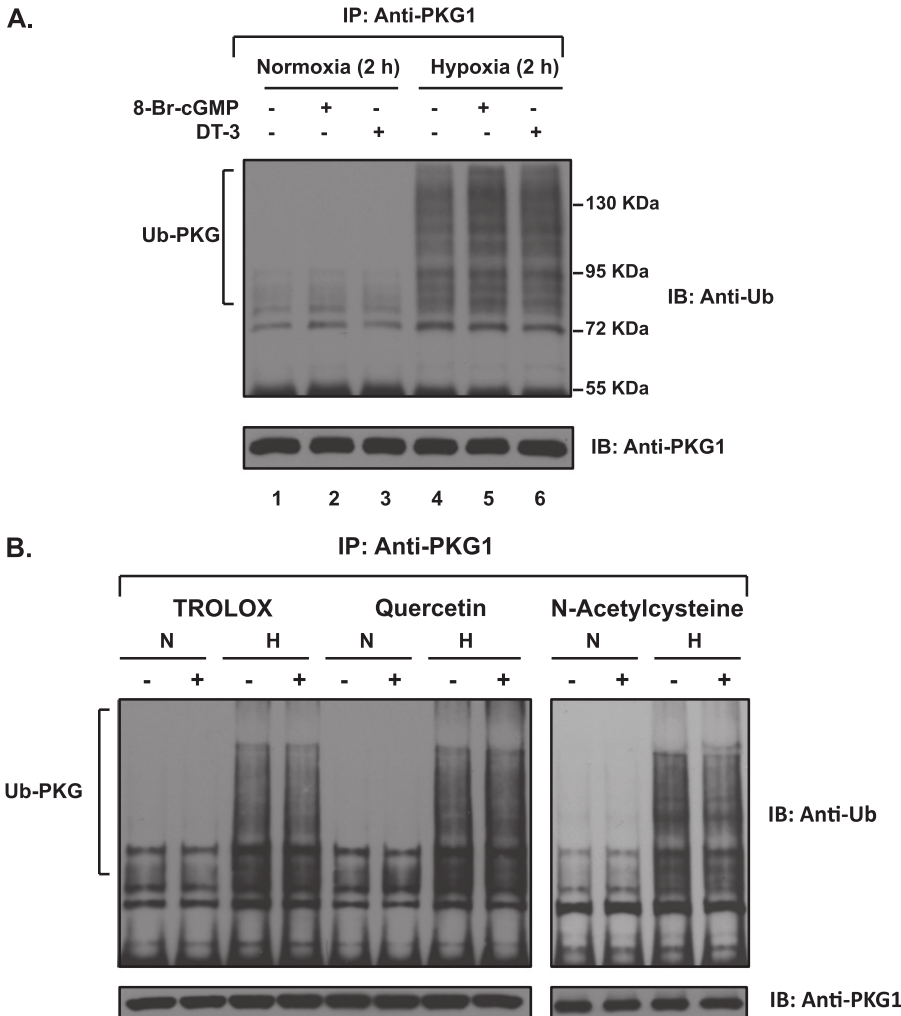


**Figure 5.** Reoxygenation reverses acute hypoxia-induced PKG1 ubiquitination. FPASMCs were exposed to hypoxia for 2 hours and transferred to normoxia for an additional 2 hours. Cell lysates were immunoprecipitated with PKG1 antibody and analyzed according to Western blotting, using anti-ubiquitin monoclonal antibody, and were reprobed with PKG1 antibody. The experiment was repeated three times, and a representative blot is shown. Duration of treatment time in hours is indicated. H, hypoxia (3% O<sub>2</sub>); N, normoxia (21% O<sub>2</sub>). Hypoxic cells that were reoxygenated contained no ubiquitinated PKG1 (Ub-PKG), similar to normoxic cells that had never been exposed to hypoxia.

SMCs is in direct contrast with, and kinetically different from, the observed down-regulation of PKG1 protein concentrations after the chronic (24-hour) activation of PKG1 in murine aortic SMCs by the cGMP analogue under normoxic conditions, as described elsewhere (20). The effect of chronic exposure to cGMP was dependent on PKG kinase activity, and has been attributed to autophosphorylation of the protein (20). Gao and colleagues reported a decrease in PKG1 protein amounts and activity when pulmonary veins were chronically (for up to 20 h) exposed to nitric oxide and elevated concentrations of cGMP (21). They found that the elevated concentrations of cGMP were exerting a negative feedback effect on concentrations of PKG1 (21). The decrease in PKG1 protein expression and activity was prevented by the administration of [1H-[1,2,4]oxadiazolo-[4, 3-a]quinoxalin-1-one] (ODQ), a sGC inhibitor or PKG inhibitor.

The specificity of ubiquitination of PKG1 during hypoxia in pulmonary vascular smooth muscle, compared with systemic vascular smooth muscle, is consistent with the physiologic response of pulmonary vessels to hypoxia, namely, vasodilatation in cerebral arteries versus vasoconstriction in pulmonary arteries (22, 23). Decreased PKG kinase activity during hypoxia augments the vasoconstrictor response of pulmonary vessels to hypoxia, especially in fetal pulmonary vessels (8, 24, 25). Cerebral artery SMCs express remarkably lower amounts of PKG1 (Figure 2) (26), and drastically reduced ubiquitinated PKG1 was noted during acute hypoxia, compared with pulmonary vascular SMCs. The regulation of the nitric oxide-cGMP pathway in cerebral vessels in hypoxia is most likely non-PKG1-mediated. Rather, a role for soluble guanylate cyclase was recently described (27). Thus the observed heterogeneity of the ubiquitin modification of PKG1 between cerebral and pulmonary vascular SMCs is consistent with their physiologic responses to acute hypoxia.

Previously, we reported that the attenuation of the NO-cGMP-PKG-dependent vasorelaxation pathway in hypoxia is related to the increased generation of reactive species. Reactive species led to the formation of nitrated PKG1 protein and decreased PKG1 activity in both intact pulmonary vessels and isolated cultured SMCs in acute hypoxia (11). Concentrations



**Figure 6.** Hypoxia-induced ubiquitin modification of PKG1 is unaffected by the endogenous activation or inhibition of its kinase activity or by common scavengers of reactive oxygen species or peroxynitrite. (A) FPASMCs were exposed to hypoxia for 2 hours, with or without the cell-permeable PKG1 inhibitor DT-3 (5  $\mu$ M), or the phosphodiesterase-resistant activator 8-bromo (Br)-cGMP (0.5 mM). The cell lysates were immunoprecipitated with PKG1 and assessed by Western blotting, using an anti-ubiquitin monoclonal antibody. The blots were also reprobated with PKG1 antibody. The level of PKG activity exerted no effect on the accumulation of ubiquitinated PKG1 (Ub-PKG) during acute hypoxia. (B) FPASMCs were exposed to hypoxia or normoxia for 3 hours, with or without 0.2 mM trolox, 0.1 mM quercetin, or 10 mM N-acetylcysteine. Cells were preincubated for 30 minutes in normoxia (21% oxygen and 5% CO<sub>2</sub>) with the different scavengers before exposure to hypoxia (3% O<sub>2</sub> and 5% CO<sub>2</sub>) in the presence of the same scavenger. The cell lysates were immunoprecipitated with PKG1 and assessed by Western blotting, using an anti-ubiquitin monoclonal antibody. The blots were also reprobated with PKG1 antibody. A representative blot from 3–4 experiments is shown.

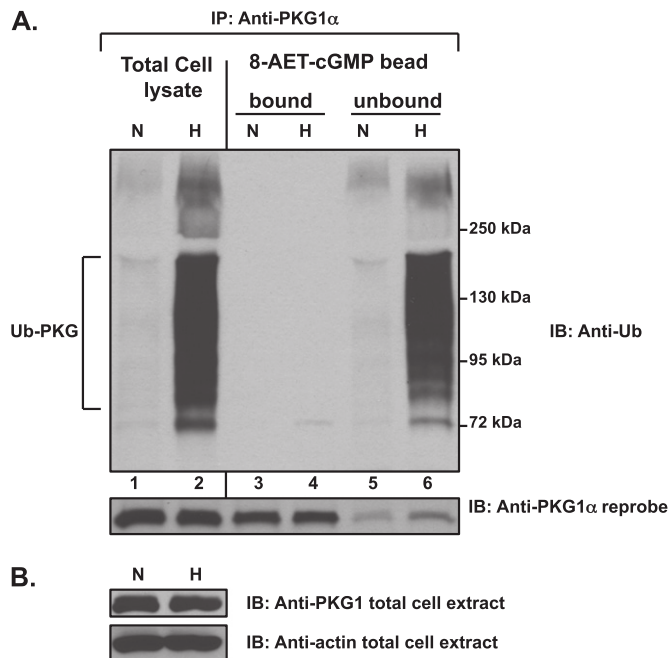
of PKG1 were markedly reduced (by 30%) after 16 hours of hypoxic treatment of pulmonary artery SMCs (Figure 4), a mechanism described earlier in freshly isolated ovine fetal pulmonary vessels and isolated ovine fetal pulmonary-vein SMCs (10, 11). We previously demonstrated that scavengers of reactive species and antioxidants restored most of the vascular dilation responses to cGMP in pulmonary veins during acute hypoxia (30 minutes), and we proposed that the ROS/reactive nitrogen species-induced nitration of PKG in hypoxia had resulted in the decreased dilation responses to cGMP during hypoxia (11). However, the treatment of FPASMCs with several known scavengers of ROS or peroxynitrite (*N*-acetylcysteine, trolox, or quercetin) did not block hypoxically induced PKG1 ubiquitination, suggesting that this modification occurs in a reactive species-independent manner (Figure 6B). Therefore, unlike the nitration of PKG, which was dependent on the generation of reactive oxygen and nitrogen species during hypoxia, the polyubiquitination of PKG1 was not mediated by reactive species. The interrelationship, if any, between PKG1 nitration and PKG1 ubiquitination during hypoxia remains to be determined.

Our data also demonstrated that hypoxically induced PKG1 polyubiquitination alters the ability of PKG1 to bind 8-AET-cGMP, a cGMP analogue, which efficiently binds cGMP-dependent PKG1 and PKG1-associated proteins (19, 28). 8-AET-cGMP efficiently bound native, unmodified PKG1 $\alpha$  in our experiments (Figure 7). We previously reported that the peroxynitrite-induced tyrosine nitration of PKG1 decreased the cGMP-dependent activation of this enzyme, but not its basal

activity (11). In these experiments, the inability of 8-AET-cGMP to bind to ubiquitin-modified PKG1 means that the cGMP-mediated activation of this enzyme would be greatly impaired. This is consistent with the observed shift in concentration required to induce 50% relaxation for 8-bromo-cGMP, from 30  $\mu$ M to 90  $\mu$ M, when isolated pulmonary vessels were exposed to acute hypoxia (11). A systematic study with ubiquitin-modified PKG1 species is necessary to confirm its cGMP-dependent activation and conformational effects.

Interestingly, our data revealed that Ub-PKG is accumulated transiently during hypoxia (for up to 4 hours), suggesting that this modified PKG1 is degraded or de-ubiquitinated with time, perhaps as part of an adaptive process. To confirm further that the ubiquitination of PKG occurs only during acute hypoxia and not prolonged hypoxia, we measured the ubiquitination of PKG1 in pulmonary arteries isolated from fetal lungs that were isolated in a hypoxic chamber, so that they would remain hypoxic and not become oxygenated (as the fetal pulmonary artery SMCs cultured in room air were). No ubiquitinated PKG1 was evident in the fetal arteries. Similarly, normoxic newborn arteries contained little ubiquitinated PKG1. Newborn SMCs, similar to fetal SMCs, responded to acute hypoxia by accumulating ubiquitinated PKG1.

Specific Ub ligases that regulate the proteolysis of substrates in hypoxia maintain cellular adaptation and homeostasis, namely, seven in absentia homolog 2 (*Drosophila*), Siah2, which binds a kinase anchor protein 1 (AKAP1) 21-regulating protein kinase A function (29); the SMAD-specific E3 ubiquitin protein ligase 1



**Figure 7.** Hypoxia-induced PKG1 ubiquitination hinders its binding to 8-(2-aminoethyl)thioguanosine-3',5' (AET)-cGMP. Cell extracts were prepared from FPASMCs exposed to hypoxia (3% O<sub>2</sub>, 3 hours) in buffer A, as described in MATERIALS AND METHODS. (A) The extracts were incubated with 8-AET-cGMP agarose beads. After washing the beads, the bound proteins were eluted with 25 mM cGMP. The total cell lysates, namely, the bead "bound" and "unbound" proteins, were immunoprecipitated with PKG1 $\alpha$  antibody and assessed by Western blotting, using an anti-ubiquitin antibody. The blots were stripped and re-probed with PKG1 $\alpha$  antibody. (B) The cell extracts were probed for PKG1 and actin. All experiments were repeated 3–4 times. A representative blot is shown. Ubiquitinated PKG1 $\alpha$  (Ub-PKG) from hypoxically exposed cells exhibited defective binding to 8-AET-cGMP, compared with native, unmodified PKG1 $\alpha$ .

(Smurf1) regulation of bone morphogenic protein (BMP) receptors in hypoxia-induced pulmonary hypertension (30); and the S-phase kinase-associated protein 2 (Skp2) regulation of p27kip1 in cGMP-induced SMC proliferation (31). Thus the identification of the hypoxia-induced E3 Ub-ligase involved in the modification of PKG1 and the specific PKG1 lysine residues involved would facilitate the delineation of this mechanism. Much remains to be known in terms of the acute effects of hypoxia on the Ub conjugation of PKG1 and the subcellular targeting of PKG1 isoforms. Because PKG1 controls diverse cellular functions *in vivo*, understanding the regulation of this protein by changes in oxygen tension via ubiquitin modification will constitute an important step toward understanding the pathophysiology of pulmonary vascular diseases.

In conclusion, we have demonstrated a new phenomenon wherein acute hypoxia rapidly induces the conjugation of multiple ubiquitin molecules to PKG1 protein, leading to an enhanced accumulation of Ub-PKG, affecting its ligand affinity. Such a modification may be partly responsible for the inhibition of the NO-cGMP-PKG1 signaling pathway and decreased vaso-relaxation seen in hypoxia. Our findings may have important implications in pulmonary vascular SMC contractility and the hypoxic pulmonary vascular hypertensive response.

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