Regulation of cGMP-dependent protein kinase-mediated vasodilation by hypoxia-induced reactive species in ovine fetal pulmonary veins

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Negash S, Gao Y, Zhou W, Liu J, Chinta S, Raj JU. Regulation of cGMP-dependent protein kinase-mediated vasodilation by hypoxia-induced reactive species in ovine fetal pulmonary veins. Am J Physiol Lung Cell Mol Physiol 293: L1012–L1020, 2007. First published July 6, 2007; doi:10.1152/ajplung.00061.2007.—We previously reported that hypoxia attenuates cGMP-dependent protein kinase (PKG)-mediated relaxation in pulmonary vessels (Am J Physiol Lung Cell Mol Physiol 279: L611–L618, 2003). To determine whether hypoxia-induced reactive oxygen and nitrogen species (ROS and RNS, respectively) may be involved in the downregulation of PKG-mediated relaxation, ovine fetal intrapulmonary veins were exposed to 4 h of normoxia or hypoxia, with or without scavengers of ROS [N-acetylcysteine (NAC)] or peroxynitrite (quercetin and Trolox) and preconstricted with endothelin-1. Hypoxia decreased the relaxation response to 8-bromo-cGMP, PKG protein expression, and kinase activity and increased tyrosine nitration in PKG. However, ROS and RNS scavengers prevented these changes. To determine whether increased PKG nitration diminishes PKG activity, pulmonary vein smooth muscle cells (PVSMC) were exposed to shorter-term (30 min) hypoxia, which increased PKG nitration and decreased PKG activity but did not alter PKG protein expression. Increased dihydro-2,7-dichlorofluorescein diacetate (DCFH2-DA) fluorescence in PVSMC after 4 h or 30 min of hypoxia was not observed in the presence of NAC, quercetin, or Trolox, suggesting increased ROS and RNS production. Increased PKG nitration and the associated decrease in PKG activity in PVSMC after 30 min of hypoxia were also reversed on reoxygenation. The consequences of PKG nitration were assessed by exposure of purified PKG-I to peroxynitrite, which caused increased 3-nitrotyrosine immunoreactivity and inhibition of kinase activity. Our data suggest that, after 30 min of hypoxia, reversible covalent modification of PKG by hypoxia-induced reactive species may be an important mechanism by which the relaxation response to cGMP is regulated. However, after 4 h of hypoxia, PKG nitration and decreased PKG expression are involved.

hypoxic vasoconstriction; vascular smooth muscle; protein kinase G

GUanosine 3′,5′-cyclic monophosphate (cGMP)-dependent protein kinase (PKG) is a serine/threonine kinase that plays an important role in the regulation of vasomotor control, possibly through attenuation of cGMP-dependent protein kinase (PKG)-mediated vasodilation, leading to speculation that ROS may serve as second messengers mediating responses to hypoxia in a variety of species (12, 21, 47, 49, 52, 56, 62). We previously reported that hypoxia attenuates cGMP-mediated relaxation in pulmonary arteries and veins of fetal lambs, primarily through attenuation of cGMP-dependent protein kinase (16), a key enzyme in vasodilation induced by endothelium-derived NO (EDNO) and other agents that elevate cGMP levels (14, 15, 36, 38). The decrease in PKG activity induced by hypoxia was more prominent in the pulmonary veins. However, it remains unclear how functional and transcriptional responses to hypoxia are mediated.

Hypoxia has been shown to induce generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in pulmonary vascular SMC (PVSMC), leading to speculation that ROS may serve as second messengers mediating responses to changes in PO2 (26, 37, 58). Here we explore the hypothesis that attenuated cGMP-PKG-mediated relaxation in hypoxia is related to increased generation of reactive species in PVSMC.

MATERIALS AND METHODS

Reagents

Indomethacin, nitro-l-arginine, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), quercetin, 3-morpholinosydnonimine hydrochloride (SIN-1), Trolox (l-(+)-6-hydroxy-2,5,7,8-tetramethyl-chro-
man-2-carboxylic acid), and all other chemicals (unless otherwise specified) were obtained from Sigma-Aldrich (St. Louis, MO). 8-Bromo-cGMP (8-BrcGMP) was purchased from Axxora LLC/Biolog Life Science Institute (San Diego, CA), endothelin-1 from American Peptide (Sunnyvale, CA), dihydro-2,7-dichlorofluorescein diacetate (DCFH$_2$-DA) from Molecular Probes (Eugene, OR), and peroxynitrite (ONOO$^-$) from Calbiochem (La Jolla, CA). Indomethacin (10$^{-7}$ M) was prepared in equal molar Na$_2$CO$_3$; this concentration of Na$_2$CO$_3$ did not significantly affect the pH of the solution in the organ chamber. Other drugs were prepared using distilled water.

Experimental Protocols

Effect of 4 h of hypoxia on PKG-mediated relaxation in ovine fetal pulmonary veins: role of reactive species. The relaxation responses of isolated intrapulmonary veins of fetal lambs, after 4 h of hypoxia, with and without scavengers of reactive species, was determined.

PREPARATION OF ISOLATED SEGMENTS OF INTRAPULMONARY VESSELS FROM FETAL LAMBS. Pregnant ewes (142–148 gestational days; Nebeker Ranch, Lancaster, CA) were killed with an overdose of pentobarbital sodium, and the fetus was removed and killed with an overdose of pentobarbital sodium. Fetal lungs were immediately removed, and fourth-generation (1.5–2 mm) pulmonary veins were dissected free of parenchyma and cut into 3-mm-long rings in ice-cold modified Krebs-Ringer bicarbonate buffer (in mM: 118.3 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 25.0 NaHCO$_3$, and 11.1 glucose). For each experiment involving pulmonary vessels, n represents the number of animals. One vessel ring from each animal was incubated with ROS/RNS scavengers before it was exposed to hypoxia or normoxia.

ISOLATED VESSEL TENSION STUDIES IN AN ORGAN CHAMBER. Vessel rings were suspended in organ chambers filled with 10 ml of Krebs-Ringer bicarbonate buffer maintained at 37°C and aerated with a normoxic (20% O$_2$-5% CO$_2$-balance N$_2$, 140 mmHg PO$_2$ in buffer) or hypoxic (0% O$_2$-95% N$_2$-5% CO$_2$, 30 mmHg PO$_2$ in buffer) gas mixture for 4 h with or without N-acetylcyesteine (NAC), quercetin, or Trolox, preconstricted with 3 × 10$^{-9}$ M endothelin-1 (55), and exposed to increasing concentrations of 8-BrcGMP. The concentration-response curves to 8-BrcGMP were constructed in a cumulative fashion. To eliminate the possible involvement of endogenous prostanoids and EDNO, 10$^{-5}$ M indomethacin (an inhibitor of cyclooxygenase) and 10$^{-4}$ M nitro-l-arginine (an inhibitor of NO synthase) were included in the medium and present throughout the experiments. Two stirrups, which were passed through the lumen, suspended each ring. One stirrup was anchored to the bottom of the organ chamber, and the other was connected to a strain gauge (model FT03C, Grass Instrument, Braintree, MA) for the measurement of isometric force (15, 16).

At the beginning of the experiment, each vessel ring was stretched incrementally to its optimal resting tension; i.e., until the active contraction of the vessel ring to 100 mM KCl reached a plateau. Then the vessels were allowed to equilibrate for 30 min.

Relaxation of pulmonary veins to 8-BrcGMP was determined after 4 h of hypoxia (30 mmHg PO$_2$) or normoxia (140 mmHg PO$_2$). Experiments were carried out in the presence of 10$^{-5}$ M indomethacin + 10$^{-4}$ M nitro-l-arginine to exclude the involvement of endogenous NO and prostaglandins. To determine whether the diminished relaxation observed under hypoxia is mediated by ROS, relaxation responses to 8-BrcGMP were obtained again with the addition of a nonspecific ROS scavenger (e.g., 10$^{-2}$ M NAC) or scavengers more selective for ONOO$^-$ (e.g., 10$^{-4}$ M quercetin and 2 × 10$^{-4}$ M Trolox) (44) to the vessel bath during hypoxia.

Effect of 4 h of hypoxia on PKG kinase activity in ovine fetal pulmonary veins: role of reactive species. PKG KINASE ACTIVITY ASSAY. As described for the vessel tension studies, PKG kinase activity was measured in intrapulmonary veins incubated in normoxia or hypoxia for 4 h in the presence or absence of ROS scavengers. Isolated pulmonary veins of near-term fetal lambs were incubated in Krebs-Ringer bicarbonate buffer maintained at 37°C and aerated with a normoxic (20% O$_2$-5% CO$_2$-balance N$_2$, 140 mmHg PO$_2$ in buffer) or hypoxic (0–1% O$_2$-95% N$_2$-5% CO$_2$, 30 mmHg PO$_2$ in buffer) gas for 4 h with or without 10$^{-2}$ M NAC.

Pulverized, quick-frozen tissue was then sonicated in a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 2 mM diethy- theitol, 1 mM isobutylmethylxanthine, 100 μM nitro-l-arginine, and 10 μM indomethacin and centrifuged at 13,000 g for 10 min at 4°C. Supernatants were assayed for PKG activity by measurement of the incorporation of [32P]ATP into a specific PKG substrate, BPDEtide (Bachem, King of Prussia, PA), as described by us previously (16). Aliquots (20 μl) of tissue extract were added to a mixture (50 μl total volume) containing 50 mM Tris-HCl (pH 7.4), 20 mM MgCl$_2$, 0.1 mM isobutylmethylxanthine, 10 μM indomethacin, 100 μM nitro-l-arginine, 150 μM BPDEtide, 1 μM PKI (a synthetic PKA inhibitor; Peninsula Laboratories, Belmont, CA), and 0.2 mM [γ-32P]ATP (3,000 Ci/mmol specific activity). The mixture was incubated at 30°C for 10 min in the presence or absence of 3 μM exogenous cGMP. The constitutive kinase activity is measured in the absence of added cGMP, and the addition of cGMP elicits the cGMP-stimulated PKG kinase activity. For termination of the reaction, 40-μl aliquots of mixture were spotted on phosphocellulose filter papers (2 × 2 cm; catalog no. F81, Whatman), which were placed in ice-cold 75 mM phosphoric acid. The filter papers were washed, dried, and counted in a liquid scintillation counter. Assays were performed in triplicate with appropriate controls. Control counts were subtracted, and remaining counts indicate PKG activity, which is expressed as picomoles of [32P] incorporated into PKG substrate per minute per milligram protein.

Effect of 4 h of hypoxia on PKG protein expression in ovine fetal pulmonary veins. WESTERN BLOT ANALYSIS. Tissue extracts were prepared from isolated pulmonary veins of near-term fetal lambs incubated in Krebs-Ringer bicarbonate buffer maintained at 37°C and aerated with a normoxic (20% O$_2$-5% CO$_2$-balance N$_2$, 100–140 mmHg PO$_2$ in buffer) or hypoxic (0–1% O$_2$-95% N$_2$-5% CO$_2$, 30 mmHg PO$_2$ in buffer) gas for 4 h in the presence or absence of NAC in RIPA buffer containing EDTA-free Complete (Roche) protease inhibitor cocktail. Tissue extracts (20 μg) were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membranes. Immunodetection was performed using an anti-PKG-I polyclonal antibody (Stressgen, Victoria, BC, Canada), which detects the α-isoform (75 kDa) and β-isoform (78 kDa) of PKG-I, and developed by a chemiluminescence detection method (ECL, Amersham). Chemiluminescence signals on X-ray films were quantified by densitometry.

Effect of 4 h of hypoxia on tyrosine nitration of PKG: 3-nitrotyrosine immunodetection. Cell extracts were prepared from intrapulmonary veins exposed to normoxia or hypoxia, homogenized in a lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and EDTA-free Complete protease inhibitor cocktail), precleared with protein G-Sepharose beads (Amersham Biosciences), and incubated with anti-PKG antibody-protein G-Sepharose bead complex overnight at 4°C with rotation. The antibody-bead complex was sedimented by centrifugation, washed three times with lysis buffer, resuspended in SDS-PAGE loading buffer, and heated for 5 min at 95°C. Western blotting was carried out as described above using a monoclonal anti-nitrotyrosine (NT) antibody (Cayman Laboratories). Protein concentration was determined with the Bradford method, with BSA used as a standard.

Production of reactive species in ovine fetal PVS MC in hypoxia. To determine whether ROS were generated in the pulmonary veins during hypoxia, we prepared PVS MC in culture for these experiments.

PVS MC. Cultured SMC from ovine fetal intrapulmonary veins (4th–5th generation) were used to assess direct effects of hypoxia on ROS production, PKG activity, and PKG. PVS MC were isolated by
enzymatic dissociation as previously described (45) and cultured in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO2 atmosphere at 37°C. Secondary cultures were obtained by serial passages after the cells were harvested with 0.05% trypsin and 0.53 mM EDTA (GIBCO-BRL/Invitrogen) and resuspended in fresh DMEM containing 10% FBS and antibiotics. Identity of SMC was determined with an SMC-specific monoclonal antibody (Sigma), and it was ascertained that SMC cultures were devoid of endothelial cells and fibroblasts. Only SMC were used. Cell phenotype did not change from passage 4 to 6 as determined by the expression of α-smooth muscle actin and myosin heavy chain proteins; nor did the amount of PKG protein or kinase activity change from passage 4 to 6.

Cells exposed to hypoxia were serum starved for 24 h in DMEM with 0.3% FBS and transferred to a Billups-Rothenberg chamber. The chamber was flushed with hypoxic gas (2% O2-5% CO2-balance N2), sealed, and placed in a 37°C incubator for 30 min or 4 h.

ROS detection with DCFH2-DA. ROS and RNS production during hypoxia was measured using DCFH2-DA, which diffuses passively through the cellular membrane and is cleaved by cellular esterases to DCFH2. In the presence of ROS, DCFH2 can be oxidized to form the fluorescent product 2′,7′-dichlorofluorescein (DCF). An increase in DCF fluorescence, therefore, indicates an increase in intracellular concentrations of ROS. Briefly, PVSMC were plated onto a 96-well plate, grown to 90% confluence, serum starved overnight, and then preincubated for 30 min with control medium or scavengers of reactive species [1 × 10⁻⁵ M NAC (a nonspecific ROS scavenger), 1 × 10⁻⁴ M quercetin (an ONOO⁻ scavenger), or 2 × 10⁻⁴ M Trolox (an ONOO⁻ scavenger)] before 4 h of hypoxia or 30 min of exposure to SIN-1. DCFH2-DA (5 μM) was added to cells 30 min before measurement of fluorescence. Fluorescence was monitored at 503-nm excitation and 523-nm emission. Background fluorescence from non-DCFH2-DA-treated cells was subtracted.

Effect of 30 min of hypoxia on PKG protein nitration and PKG kinase activity. To determine whether PKG protein nitration and attenuation of PKG kinase activity would occur with hypoxia in the absence of a decrease in PKG protein expression, we performed experiments with cultured PVSMC exposed to 30 min of hypoxia and measured PKG protein expression, protein nitration, protein kinase activity, and PKG activity.

Confirmation that nitration of pure PKG protein by ONOO⁻ leads to decreased PKG kinase activity. PKG purified from bovine lung (Promega, Madison, WI) was suspended in 50 mM KH₂PO₄ (pH 7.4) and 100 μM diethylenetriamine pentaacetic acid and incubated in increasing concentrations of ONOO⁻ in 0.1% NaOH solution at room temperature with rotation for 5 min. Aliquots of PKG modified with ONOO⁻ were assayed for constitutive and cGMP-stimulated kinase activity as described above.

Presence of nitrite and nitrate in the absence of endothelium in pulmonary vessels. Since the effects of ONOO⁻ were observed in the isolated vessels, even in the presence of an inhibitor of endothelial NO synthase (nNOS) and in cultured PVSMC, as evidenced by nitration of PKG protein during hypoxia, an alternative source of NO in vessels was determined by the measurement of nitrite and nitrate in isolated endothelium-denuded ovine fetal pulmonary veins.

The nitrate and nitrite levels were determined using a nitrate/nitrite fluorometric assay kit (Cayman Chemical) (43). Briefly, pulverized frozen vessels were homogenized in PBS (pH 7.4) and centrifuged at 10,000 g for 20 min. The supernatant was centrifuged at 100,000 g for 30 min and then subjected to ultrafiltration through a 30-kDa-molecule cut-off filter to remove proteins, which may interfere with the assay. The filtrate was subsequently assayed for nitrite following the manufacturer’s protocol. Total nitrite/nitrate content was assayed by first reducing nitrate to nitrite by nitrate reductase in the presence of essential cofactors NADPH and FAD. Nitrite reacts with 2,3-diaminonaphthalene under acidic conditions to form a fluorescent com-

Statistical Analysis

Values are means ± SE. Student’s t-test for unpaired observations was used for comparison of mean values of two groups. Student’s t-test for paired observations was used for comparison of mean values of the same group before and after stimulation. Mean values of more than two groups were compared using one-way ANOVA with post hoc testing of multiple comparisons by the Student-Newman-Keuls test. All analyses were performed using a commercially available statistics package (SigmaStat, Jandel Scientific, San Rafael, CA).

RESULTS

ROS and RNS Play a Role in Hypoxia-Induced Attenuation of PKG-Mediated Relaxation in Pulmonary Veins

Under normoxia (140 mmHg PO₂), 8-BrcGMP induced concentration-dependent relaxation of pulmonary veins (Fig. 1). However, the relaxation was attenuated under hypoxia, as observed previously (16). Scavenging ROS using a nonspecific scavenger such as NAC restored the relaxation response to cGMP in pulmonary veins; however, ROS scavengers that are more selective for ONOO⁻ (quercetin and Trolox) appeared slightly more effective than the general ROS scavenger NAC. The EC₅₀ for cGMP, i.e., the concentration of cGMP required to induce 50% relaxation in the vessels, was ~30 μM in normoxia. This value increased to ~90 μM in hypoxia, but the presence of NAC, Trolox, or quercetin in hypoxia reduced the EC₅₀ of cGMP in normoxic levels.
ROS and RNS Generated Over 4 Hours of Hypoxia Are Involved in the Decrease in PKG-I Kinase Activity in Pulmonary Veins

The constitutive PKG kinase activity, in the absence of exogenous cGMP, in pulmonary veins under normoxia or hypoxia was significantly lower than PKG kinase activity in the presence of exogenous cGMP (Fig. 2). This observation is consistent with the ability of cGMP to bind and activate PKG (14). Constitutive and cGMP-stimulated PKG kinase activities were reduced in hypoxia compared with normoxia, but the major effect of hypoxia was on the cGMP-stimulated kinase activity (67 ± 4.8 and 107 ± 1.4 pmol·min⁻¹·mg protein⁻¹ in hypoxia and normoxia, respectively), suggesting that residues involved in cGMP binding may be susceptible to hypoxia-induced posttranslational modification. The inhibition was reversed in the presence of NAC, implying a role for ROS and RNS in the modulation of PKG kinase activity. NAC had no significant effect on PKG activity in normoxia.

PKG Expression Is Decreased and Covalent Modification of PKG-I Is Increased by ROS and RNS After 4 Hours of Hypoxia in Pulmonary Veins

The downregulation of PKG activity in hypoxia suggests that ROS and RNS generated during hypoxia might play a role in the downregulation of PKG protein and/or activity. Immunoblot analyses indicate that PKG protein expression is decreased in vessels exposed to 4 h of hypoxia (Fig. 3A). However, this decrease in protein expression was prevented when hypoxia-induced reactive species were scavenged with NAC, suggesting that ROS and RNS may play a role in the regulation of PKG protein levels in hypoxia. Next, we considered the possibility that, in addition to regulating PKG protein levels, hypoxia-induced ROS and RNS may covalently modify PKG and modulate its activity. PKG tyrosine nitration was assessed by immunoprecipitating PKG from homogenates of

Fig. 2. PKG kinase activity in isolated pulmonary veins after 4 h of normoxia or hypoxia in the presence or absence of 1 × 10⁻² M NAC. (–)cGMP, without cGMP; (+)cGMP, with 3 × 10⁻⁶ M cGMP. Values are means ± SE (n = 4). *Significantly different from respective (–)cGMP control (P < 0.05). †Significantly different from normoxia + NAC, hypoxia + NAC, or normoxia (P < 0.05). PKG activity is presented as a percentage, with normoxia (+)cGMP set at 100%.

Fig. 3. Hypoxia-induced reactive oxygen and nitrogen species (ROS and RNS)-mediated modulation of PKG-I protein expression and covalent modification in pulmonary veins. A: PKG and actin expression in homogenates of fetal intrapulmonary veins exposed to normoxia, hypoxia, or hypoxia + NAC. Top: representative blots of PKG and actin expression in homogenates exposed to normoxia (control, lane 1), hypoxia (lane 2), or hypoxia + NAC (lane 3). Bottom: densitometric quantification of PKG protein normalized to actin. AU, arbitrary units. Values are means ± SE (n = 3). *Significantly different from control or hypoxia + NAC (P < 0.05).

B: detection of 3-nitrotyrosine (3-NT) in PKG immunoprecipitated from homogenates of pulmonary veins exposed to normoxia or hypoxia for 4 h and probed for 3-NT using a monoclonal antibody against protein 3-NT (Cayman Laboratories). Top: representative blots of 3-NT in PKG immunoprecipitated from homogenates of pulmonary veins exposed to normoxia or hypoxia for 4 h and probed for 3-NT using a monoclonal antibody against protein 3-NT (Cayman Laboratories). Top: representative blots of 3-NT in PKG immunoprecipitated from homogenates of pulmonary veins exposed to normoxia (lane 1) or hypoxia (lane 2). Bottom: densitometric quantification of 3-NT in immunoprecipitated PKG normalized to PKG expression in lysates of respective samples. Values are means ± SE (n = 3). *Significantly different from normoxia (P < 0.05).
pulmonary veins incubated in normoxia or hypoxia for 4 h and probing for 3-NT immunoreactivity (Fig. 3B). Although total PKG protein expression was decreased in hypoxia (Fig. 3A), PKG nitration increased significantly in hypoxia, indicating that ONOO− production is augmented in hypoxia, leading to the posttranslational modification of PKG.

**ROS Generation in PVSMC During 4 Hours and 30 Minutes of Hypoxia**

ROS formation in hypoxia was measured using the ROS indicator DCFH2-DA. To assess the generation of ROS and, more specifically, ONOO−, PVSMC were exposed to normoxia or hypoxia in the presence or absence of 10−2 M NAC, 1 × 10−4 M quercetin, or 2 × 10−4 M Trolox (Fig. 4A). Exposure to 4 h of hypoxia induced a significant increase in DCF fluorescence, but this increase was attenuated in the presence of a general ROS scavenger, NAC, or scavengers of ONOO− (quercetin or Trolox), indicating the generation of ROS as well as ONOO− in hypoxia. With 30 min of exposure to hypoxia the results were similar, except for a smaller hypoxia-induced increase in DCF fluorescence (Fig. 4B). Furthermore, the increase in DCF fluorescence intensity on exposure to 30 min of hypoxia was inhibited by the same set of ROS and RNS scavengers used in the 4-h hypoxia experiments (Fig. 4A), suggesting that ROS and RNS in general, and ONOO− in particular, are generated in response to more acute hypoxia.

To determine the consequence of a more acute (30-min) exposure to hypoxia on PKG kinase activity in the absence of a decrease in PKG protein levels, the expression and posttranslational modification of PKG protein, and the reversibility of these effects (Fig. 5), PVSMC were exposed to normoxia or hypoxia for 30 min or allowed to recover in normoxia for 30 min after 30 min of hypoxia. As observed in 4-h hypoxia experiments, ROS and RNS production increased in PVSMC exposed to hypoxia (30 mmHg Po2) for 30 min (Fig. 5A). However, if hypoxia was followed by 30 min of recovery in normoxia (140 mmHg Po2), the amount of ROS produced was not significantly different from that produced under normoxia, indicating that ROS are generated in PVSMC in a PO2-dependent and reversible manner. Similarly, PKG activity was lower in PVSMC exposed to 30 min of hypoxia than in PVSMC exposed to hypoxia and allowed to recover in normoxia (Fig. 5B). Furthermore, in cells that were exposed to hypoxia, allowed to recover in normoxia, and then reexposed to hypoxia, PKG kinase activity was reduced, suggesting that PKG kinase activity is inhibited by hypoxia in a PO2-dependent, reversible manner. Contrary to the decrease in PKG protein that was observed in PVSMC exposed to 4 h hypoxia (Fig. 3B), 30 min of hypoxia had no significant effect on PKG protein expression in PVSMC (Fig. 5C). However, 30 min of hypoxia led to increased tyrosine nitration in PKG, which was reversed when cells are allowed to recover in normoxia for 30 min after exposure to hypoxia (Fig. 5D). The O2-dependent modulation of ROS production, PKG nitration, and the correlated decrease in PKG kinase activity in the absence of changes in PKG protein expression suggest that ROS-dependent tyrosine nitration may serve as a signaling mechanism by which PKG kinase activity is regulated in response to changes in Po2 in vivo.

**Dose-Dependent Effect of ONOO− on PKG-Iα Covalent Modification and Kinase Activity**

We further examined the direct effect of ONOO− on PKG nitration and the resulting functional consequences by incubating purified PKG-Iα with increasing concentrations of ONOO− and assessing PKG kinase activity in the presence or absence of cGMP (Fig. 6). A dose-dependent increase in 3-NT immunoreactivity (Fig. 6, A and B) and a concomitant concentration-dependent inhibition of PKG kinase activity (Fig. 6C) by ONOO− were observed, suggesting that, in addition to their contribution to the downregulation of PKG protein expression in hypoxia, ROS and RNS likely play a role in modulating

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**Fig. 4.** ROS and RNS production in PVSMC during hypoxia. PVSMC were exposed to normoxia or hypoxia for 4 h (A) or 30 min (B) in the presence or absence of NAC, quercetin, or Trolox, and ROS and RNS levels were measured using 5 μM dihydro-2,7-dichlorofluorescein diacetate (DCFH2-DA). Values are means ± SE (n = 3), *p < 0.05 vs. corresponding normoxia control or hypoxia in the presence of ROS and RNS scavengers.
PKG regulation by hypoxia-induced reactive species

PKG kinase activity. As expected, constitutive PKG kinase activity was significantly lower in the absence than in the presence of exogenous cGMP. Constitutive and cGMP-stimulated PKG kinase activity were sensitive to ONOO\(^-\), but the effect on cGMP-stimulated kinase activity is more prominent, suggesting that one or more residues within the cGMP-binding region of PKG are susceptible to modification by ONOO\(^-\).

Presence of Nitrite in Isolated Endothelium-Denuded Vessels

ONOO\(^-\), a product of the reaction of NO with superoxide, is a potent and versatile oxidant that can react with a wide range of biological molecules, including DNA, proteins, and lipids, in physiological conditions. The increase in tyrosine nitration of PKG in fetal pulmonary veins in the presence of an eNOS inhibitor (nitro-L-arginine) prompted us to look for an alternative biological source of NO that can potentially serve as a source for ONOO\(^-\) under hypoxic conditions. Alternative biological sources of NO include nitrite and nitrate (41). We, therefore, used a fluorometric assay to determine nitrite/nitrate levels in endothelium-denuded pulmonary veins, which lack eNOS as a source of NO (Fig. 7). We detected nitrite and nitrate (7.5 ± 0.8 and 47.2 ± 1.5 nmol/mg, respectively) in these vessels.

DISCUSSION

Acute hypoxic pulmonary vasoconstriction is a physiological response whereby circulating blood is diverted from hypoxic alveoli to optimize the matching of perfusion with ventilation. In the fetus, the low-O\(_2\) environment in utero results in vasoconstriction of arteries and veins, which is more of a global hypoxic pulmonary vasoconstriction. With oxygenation, arteries and veins relax, resulting in a fall in total pulmonary vascular resistance and an increase in pulmonary blood flow. We previously reported that incubation of fetal pulmonary vessels in a physiological hypoxic medium for 20 h (P\(_{O_2}\) similar to that in utero) attenuates relaxation mediated by cGMP-dependent protein kinase (16) by downregulating PKG activity and the expression of PKG protein and mRNA. Furthermore, the effect of hypoxia on PKG expression was more prominent in pulmonary veins than in arteries. The present study was, therefore, designed to investigate the molecular mechanisms involved in the downregulation of PKG in ovine fetal pulmonary veins in physiological hypoxia (30–40 mmHg P\(_{O_2}\)). We show that hypoxia-induced downregulation of cGMP-mediated relaxation in pulmonary veins is reversed by the presence of ROS and RNS scavengers, suggesting that reactive species may play a role in maintaining hypoxic vasoconstriction in utero and contribute to mediating pulmonary vasoconstriction in response to acute decreases in P\(_{O_2}\).

Effect of 4 Hours of Hypoxia on PKG-Mediated Relaxation in Ovine Fetal Pulmonary Veins: Role of Reactive Species

We previously reported that the relaxation response of pulmonary veins exposed to 1, 2, 4, 8, and 20 h of hypoxia (30 mmHg P\(_{O_2}\)) or normoxia (140 mmHg P\(_{O_2}\)) to 8-BrcGMP reached maximal attenuation within 4 h, and the diminished relaxation to cGMP after 4 h of hypoxia was reversed after reexposure to 4 h of normoxia (16). We hypothesized that ROS produced in hypoxia may play a role in mediating this reversible process. ROS play important roles in a wide variety of cellular processes, and many tissues produce ROS during reoxygenation after hypoxia or ischemia; however, whether
ROS are formed during hypoxia remains controversial (59). In agreement with previous observations in cultured pulmonary artery SMC (26), pulmonary arteries (37), or perfused lungs (58), our findings in PVSMC support the notion that hypoxia increases ROS and RNS production. Using the ROS indicator probe DCFH2-DA and reactive species scavengers more specific for ONOO\(^{-}/H_2O_2\), we show that ONOO\(^{-}/H_2O_2\) may be generated in hypoxia. Furthermore, we show that hypoxia-induced downregulation of cGMP-mediated relaxation in pulmonary veins is reversed by the presence of the nonspecific ROS scavenger NAC, as well as the ONOO\(^{-}\)-specific scavengers quercetin and Trolox, indicating that the decrease in cGMP-mediated relaxation in hypoxia is mediated by ROS and RNS.

Effect of 4 Hours of Hypoxia on Downregulation of PKG Kinase Activity Via Decreased Protein Levels and Increased Tyrosine Nitration of PKG

We have found that the downregulation of PKG-I kinase activity during hypoxia is coupled with decrease in PKG protein expression and an increase in PKG protein nitration after 4 h of hypoxia. The presence of NAC during hypoxia, however, reverses all these effects, suggesting that ROS mediate the downregulation of PKG-I protein levels, perhaps by enhancing the susceptibility of PKG-I to degradation by the proteasome, as well as inhibiting its kinase activity. Tyrosine nitration has, in fact, been shown to enhance proteolytic degradation of nitrated proteins, favoring faster clearance (53); however, accumulation of nitrated proteins has been observed under pathological conditions and in aging (24). Thus, under conditions of prolonged low PO\(_2\) (4 h of hypoxia), diminished relaxation to cGMP may be fully explained by the decrease in PKG protein levels, but the accumulation of NT in PKG could also contribute to diminished PKG activity and PKG-mediated relaxation.

Effect of 30 Minutes of Hypoxia on Downregulation of PKG Kinase Activity Via Increased Tyrosine Nitration of PKG

In more acute (30-min) hypoxia, ROS- and RNS-mediated covalent modification of PKG, which appears to be a reversible phenomenon, occurs in the absence of changes in protein expression and can account for the hypoxia-induced decrease in PKG kinase activity. Under our experimental conditions, PKG kinase activity and relaxation to cGMP were studied in vessels pretreated with an inhibitor of NO synthases (nitro-L-arginine) to eliminate the confounding effects of endogenous EDNO; therefore, other sources of NO likely participated in the production of ONOO\(^{-}\) under hypoxia. Alternative physiological sources of NO synthase-independent NO include endogenous nitrite and nitrate (39). It has been reported that xanthine oxidase can catalyze the reduction of nitrite and/or nitrate to NO under hypoxic (35, 41, 60) and anoxic (9, 33, 34) conditions. We have found that nitrite and nitrate are present in
ovine fetal pulmonary veins. Thus it is likely that nitrite and nitrate serve as sources of NO, providing an essential substrate for ONOO\textsuperscript{−} formation in fetal pulmonary veins under hypoxic conditions.

**Effect of ROS- and RNS-Induced Tyrosine Nitration on Protein Function**

ONOO\textsuperscript{−}, a potent oxidant formed by the combination of superoxide anion and NO, can modify a number of biological molecules, including proteins, lipids, and nucleic acids. Among other modifications, ONOO\textsuperscript{−} is known to promote the nitration of tyrosine and tryptophan residues, S-nitrosylation of cysteine residues, and oxidation of methionine and cysteine residues of proteins (7, 10, 54, 57). It has also been suggested that nitration of a tyrosine residue may prevent the subsequent phosphorylation of that residue (20, 29). Alternatively, nitration of tyrosine residues may stimulate phosphorylation (8, 40) and result in constitutively active proteins. Our observation of increased PKG-I nitration in hypoxia supports the notion that ROS and RNS are generated in hypoxia, as the covalent addition of a nitro group to protein tyrosine, resulting in protein modification via 3-NT, is considered a biomarker of the generation of RNS (4, 5, 24, 50). Although a gain of function has been reported for the nitration of some proteins such as PKC-ε (3), the inhibition of function appears to be a more common consequence of protein nitration (1). In the case of PKG, increased tyrosine nitration appears to correlate with the inhibition of kinase activity.

**Reversibility of PKG Nitration**

Although the identification of a putative “denitrase” activity suggests a signaling role for protein nitration involving controlled denitration, the reversibility of protein tyrosine nitration remains to be elucidated (20, 25, 30, 31). Redox-based post-translational modification of proteins via tyrosine nitration at restricted, physiological O\textsubscript{2} concentrations allows for the dynamic regulation of protein function in a rapid and selective manner, allowing for an immediate and reversible functional response. PO\textsubscript{2}-dependent covalent modifications involving tyrosine nitration and denitration of mitochondrial proteins have recently been reported (28). The present study shows that PKG-I is reversibly nitrated and inhibited in a redox-dependent manner, and this cycle of protein nitration and denitration is analogous to protein phosphorylation and suggests that PO\textsubscript{2}-dependent tyrosine nitration may play a signaling role in modulating PKG activity, although the potential contribution of oxidative modifications different from tyrosine nitration have yet to be excluded. The impact of tyrosine nitration and oxidative modification on the stability of PKG protein also needs to be explored. Our findings suggest that hypoxia-induced reactive species may, initially, mediate their control of the NO-cGMP-PKG vasodilation pathway by covalently modifying and inactivating a key player of this pathway, PKG, and, finally, exert their control at the transcriptional, translational, and/or posttranslational levels, significantly reducing its expression and, thus, leading to prolonged vasoconstriction.

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**REFERENCES**


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