ROLE OF cGMP-DEPENDENT PROTEIN KINASE (PKG) IN REGULATION OF PULMONARY VASCULAR SMOOTH MUSCLE CELL ADHESION AND MIGRATION: EFFECT OF HYPOXIA.


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Running Head: PKG regulates SMC adhesion and migration in hypoxia.
ABSTRACT

Exposure to prolonged hypoxia can result in pulmonary vascular remodeling and pulmonary hypertension. Hypoxia induces pulmonary vascular smooth muscle cell (PVSMC) proliferation and vascular remodeling by affecting cell adhesion, migration and secretion of extracellular matrix proteins. We have previously shown that acute hypoxia decreases cGMP-dependent protein kinase (PKG) activity in PVSMC and that PKG plays a role in maintaining the differentiated contractile phenotype in normoxia. In this study, we investigated the effect of hypoxia on PVSMC adhesion and migration and the role of PKG in these functions. Ovine fetal pulmonary artery SMC were incubated in normoxia (pO$_2$ ~ 100 torr), hypoxia (pO$_2$ ~ 30-40 torr), or treated with a PKG inhibitor DT-3 for 24 h in normoxia. To further study the role of PKG in the modulation of adhesion and migration, PVSMCs were transiently transfected with a full-length PKG1$\alpha$ (PKG-GFP) or a dominant negative construct (G1$\alpha$R-GFP). Cell adhesion to extracellular matrix proteins was determined, and integrin-mediated adhesion assessed using $\alpha/\beta$ integrin-mediated cell adhesion array. Exposure to hypoxia (24h) and pharmacological inhibition of PKG1 by DT-3 significantly promoted adhesion mediated by $\alpha_4$, $\beta_1$ and $\alpha_5$$\beta_1$ integrins to fibronectin, laminin and tenacin and also resulted in increased cell migration. Likewise, inhibition of PKG by expression of a dominant negative PKG1$\alpha$ construct increased cell adhesion and migration, comparable to that induced by hypoxia. Dynamic actin reorganization associated with integrin-mediated cell adhesion is partly regulated by actin-binding protein cofilin, the (Ser3) phosphorylation of which inhibits its actin-severing activity. We found that increased PKG expression and activity is associated with decreased cofilin (Ser3) phosphorylation, implying a role for PKG in the modulation of cofilin activity and actin dynamics. Taken together, these findings identify cGMP/PKG1 signaling as central to the functional differences between PVSMC exposed to normoxia versus hypoxia.

Key Words

Smooth muscle cell; hypoxia; pulmonary artery; remodeling
INTRODUCTION

Chronic hypoxia can induce pulmonary vascular remodeling with thickening of the vascular smooth muscle coat and altered vasoreactivity, changes that result in pulmonary hypertension (7; 21). Vascular smooth muscle cell (SMC) responses to environmental stresses such as chronic hypoxia are characterized by alterations in the SMC to the synthetic phenotype, demonstrating increased proliferation, increased secretion of extracellular matrix proteins, and enhanced motility of smooth muscle cells (15).

Our laboratory has previously shown that cGMP-dependent protein kinase (PKG) plays an important role in nitric oxide-cGMP mediated relaxation in fetal pulmonary arteries and veins under normoxic conditions (8). Earlier, we reported that acute hypoxia decreases PKG activity in fetal pulmonary vascular smooth muscle. In acute hypoxia, PKG activity is significantly lower than in normoxia, both in the arteries and veins, though the effect of hypoxia is greater in veins (9). PKG has also been found to have other functions in vascular smooth muscle, including in the systemic circulation. Lincoln et al reported that PKG is essential for maintaining the contractile phenotype of aortic smooth muscle (12; 13). We have also previously shown that hypoxia-induced decreases in PKG activity in fetal pulmonary vascular smooth muscle are associated with loss of the contractile properties and the differentiated phenotype of pulmonary vascular SMC (28).

Integrins interact with extracellular matrix proteins to facilitate adherence of the contractile SMC to the extracellular matrix (15). Integrins regulate SMC functions such as adhesion, migration, and proliferation. When the SMC becomes de-differentiated, a different set of adhesion receptors are expressed in the motile, synthetic, non-contractile SMC. Disengagement of adhesion receptors is required for the synthetic and migratory activities of motile SMCs (15). In order for cells to migrate, there must be initial transient attachments at the leading edge occurring concurrently with the release of the receding edge. There is a delicate balance that determines the extent of migration. Adhesion must not be too strong to prevent movement, yet adhesion must not be too weak to provide traction (1).

In this study, we investigated the effect of hypoxia on the adhesion and migration properties of ovine fetal pulmonary artery SMC (FPASMC). We also explored the role of PKG in regulating cell adhesion and migration by studying the effects of inhibition of PKG expression and function.
METHODS

Reagents. Rabbit polyclonal anti-GFP antibody was obtained from Abcam. Rabbit polyclonal antibodies against coflin, phospho-cofilin (Ser3), VASP (A290) and phospho-VASP (Ser239) were obtained from Cell Signaling Technologies. Anti-PKG (CT) antibody was obtained from StressGen Bioreagents. All other reagents, not otherwise specified, were obtained from Sigma.

Cell culture. Ovine fetal pulmonary artery smooth muscle cells (FPASMC) were isolated and prepared as described previously. The cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 400 ng/ml amphotericin B, 160 U/ml of penicillin and streptomycin (Invitrogen). Cells were cultured in a humidified incubator with constant supply of 5% CO₂ at 37°C. For all experiments, subconfluent (50-70%) FPASMC (passage 3-6) were growth arrested in 0.3% FBS starvation media for 24 hours prior to treatment/assay.

Hypoxia and Normoxia treatments. A humidified box for cell culture (C-Chamber Series, BioSpherix) was used as a hypoxia chamber. A gas mixture of 5% CO₂ with balance N₂ was used to maintain the chamber at 2% oxygen for hypoxia (pO₂ ~30-40 torr in the media), and the oxygen concentration was monitored with an oxygen sensor (ProOxC, BioSpherix). For normoxia experiments, cells were incubated in a humidified incubator with constant supply of 5% CO₂ at 37°C (21% oxygen, pO₂ ~100 torr in the media). The pO₂ in the cell media during hypoxia was 30-40 torr and during normoxia was ~100 torr. Cells were exposed to hypoxia or normoxia for 24 hours prior to performing the experiments.

Treatment of FPASMC with PKG inhibitor in normoxia. Subconfluent cells were starved for 24 hours in 0.3% FBS-containing DMEM and treated with DT-3 (3x10⁻⁶M, Calbiochem), a membrane permeable peptide inhibitor of PKG-1α, for 24 hours and then the cells were resuspended in the presence or absence of DT-3 prior to use in adhesion assays and Boyden chamber method for migration assay.

Transient transfection of FPASMC. Plasmids encoding full-length PKG1α tagged with green fluorescent protein (PKG-GFP) or a dominant negative construct consisting of a truncation encoding the regulatory domain of PKG1α (PKG G1αR-GFP) in pEGFP-N1 vector (Clontech) were kindly provided by Dr. Darren D. Browning. The PKG G1αR-GFP truncated protein was found to dimerize with endogenous type 1 PKG and behave in a dominant negative manner (3). FPASMCs were transfected with PKG-GFP, PKG G1αR-GFP or vector alone (pEGFP-N1) using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer’s protocol. Briefly, cells were seeded at a density of ~ 6.3×10⁴/cm² in DMEM with 10% FBS and allowed to attach overnight. Transfection was performed 1 day after seeding using 2.5 µg of DNA , 2.5 µl PLUS reagent and 6.25 µl of Lipofectamin LTX per well. Cells were trypsinized and seeded onto P100 tissue culture plates 48 hours after transfection. The next day, cells were starved overnight and treated with hypoxia or normoxia for 24 h. Transfection efficiency was confirmed by western blot analysis of PKG expression and by assaying PKG kinase activity. Because of the similarity in size of GFP (~30kDa) to the deleted portion of PKG, the molecular weight of the fusion protein is 85kDa (3).
**Western immunoblot analysis.** Cell lysates were prepared from treated and untreated cells. After appropriate treatment, cells were washed with PBS, lysed with RIPA buffer (50 mM pH 7.4 Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, Boston BioProducts) containing protease inhibitor cocktail (Roche Diagnostics) and PMSF (1mM), sonicated twice on ice for 5 seconds each, centrifuged (12,000 xg, 10 min, 4°C), and supernatant stored at -80°C. Protein concentration was determined using BCA Protein Assay Kit (Pierce). Cell extracts were separated by SDS-PAGE, transferred onto nitrocellulose membranes, blocked with 5% non-fat milk in TBS-T (20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% Tween 20), and incubated at 4°C overnight with primary antibodies that were diluted in 5% BSA in TBS-T. Membranes were then washed with TBS-T and incubated with diluted secondary antibody for 1 hour at room temperature. Super signal west pico chemiluminescent substrate (Pierce) was used for signal generation. The primary antibodies used were anti-PKG type 1 (cat. no. KAS-PK005, polyclonal; dilution, 1:5,000; Stressgen, Victoria, BC, Canada); anti-VASP (cat. no. 3120, polyclonal; dilution, 1:1,000; Cell Signaling Technology, Danvers, MA); anti-phospho-VASP (Ser239) (cat. no. 3114, polyclonal; dilution, 1:1,000; Cell Signaling Technology); anti-cofilin (cat. no. 3318, polyclonal; dilution, 1:1,000; Cell Signaling Technology); anti-phospho-cofilin(Ser3) (cat. no. 3313, polyclonal; dilution, 1:1,000; Cell Signaling Technology), anti-actin antibody (cat. no. CP01, monoclonal; dilution, 1:10,000; EMD Chemicals, La Jolla, CA) and anti-GFP (cat. no. ab6556, polyclonal; dilution, 1:2000; Abcam, Cambridge, MA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies were used at 1:2000 dilution (for VASP, phospho-VASP(Ser239), cofilin, phospho-cofilin(Ser3)), 1:5000 (for PKG1) and 1:20,000 (for actin).

**PKG kinase assay.** Cultured FPASMC were rinsed with ice-cold PBS, harvested and sonicated in a buffer containing 50 mM Tris•HCl (pH 7.4), 10 mM EDTA, 2 mM dithiothreitol, 1 mM isobutylmethylxanthine, 100 μM nitro-L-arginine, and 10 μM indomethacin and centrifuged at 13,000 xg for 10 min at 4°C. Supernatants were assayed for PKG activity by measuring the incorporation of $^{32}$P from $^{\gamma}$-32P]ATP into a specific PKG substrate, BPDEtide (Bachem, King of Prussia, PA), as described by us previously (9). Aliquots (20 μl) of tissue extract were added to a mixture (total volume, 50 μl) 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 [$^{\gamma}$-32P] ATP (specific activity 3,000 Ci/mmol). The mixture was incubated at 30°C for 10 min in the presence or absence of 3 μM exogenous cGMP. In the absence of added cGMP, the constitutive kinase activity is measured and the addition of cGMP elicits the cGMP-stimulated PKG kinase activity. The reaction was terminated by spotting 40-μl aliquots of mixture on phosphocellulose papers (2 x 2 cm; P81 Whatman) and placing them 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. After subtracting control counts, counts obtained indicate PKG activity, which is expressed as picomoles of $^{32}$P incorporated into PKG substrate per minute per milligram protein.

**Adhesion assay.** To study cell adhesion to extracellular matrix (ECM) proteins, adhesion assays were performed on ECM Array Plates (Chemicon / Millipore). Each 8-well strip consists of 7 different human ECM protein-coated wells (collagen I, collagen II, collagen IV, fibronectin, laminin, tenascin, vitronectin) and one BSA-coated well (negative control). Briefly, wells were
re-hydrated with PBS for 15 min at room temperature, and the PBS was removed prior to seeding of cells. Treated FPASMCs were then harvested and plated at $3 \times 10^5$ cells per well in serum free culture medium and incubated at 37°C for 1 hour. Medium and non-adherent cells were then removed from the wells and rinsed gently with PBS, and adherent cells were fixed and stained with 0.2% crystal violet in 10% acetic acid. The dye was solubilized using a buffer of 0.1M NaH$_2$PO$_4$ and 50% ethanol, and absorbance (570nm) was measured.

**Integrin-mediated binding assays.** Integrin mediated adhesion was examined using the α/β Integrin-Mediated Cell Adhesion Array Combo kits (Chemicon / Millipore). These kits use mouse monoclonal antibodies generated against human alpha (α1, α2, α3, α4, α5, αV, and αvβ3), and beta (β1, β2, β3, β4, β6, αVβ5, and α5β1) integrins/subunits, that are immobilized onto a goat anti-mouse antibody coated micro-titer plate. The plate is then used to capture cells expressing these integrins on their cell surface. Cells were exposed to normoxia, hypoxia or normoxia in the presence of DT-3 (3 μM) for 24 h, harvested and plated at $3 \times 10^5$ cells per well in triplicate in serum free culture medium and incubated at 37°C for 1 hour. Medium and unbound cells were removed from the wells and rinsed gently twice with 200 μl assay buffer. 100 μl of cell stain solution was added to each well and incubated for 5 minutes at room temperature. The plates were washed 3 times with de-ionized water and allowed to air dry. 100 μL of Extraction Buffer was added to each well and absorbance (570nm) was measured.

**Wound-healing migration assay.** To study cell migration, a wound-healing method was used. FPASMCs were grown to confluence on 60 mm dishes. Cells were then growth arrested for 24 hours and 5mM hydroxyurea was added to prevent cell proliferation. A scratch was made in the cell monolayer, baseline images were captured, and the dishes were placed in hypoxia or normoxia. Migration was assessed at 24 hours by counting the number of cells that migrated across the scratch and normalized to scratch area. Differences among control and treated groups were expressed as percent of control (assigned 100%) and shown in bar diagrams with means ± standard error. Migration data were obtained from 3 independent wound-healing experiments.

**Boyden chamber migration assay.** Cell migration was also studied using an 8μm pore size transwell chemotaxis cell migration assay kit (Chemicon International) according to manufacturer’s protocol. Briefly, FPASMCs were first treated with normoxia, hypoxia, or normoxia with PKG inhibitor, harvested, and $5 \times 10^5$ cells were plated into each well of the migration chamber, performing each condition in triplicates. DMEM without FBS was added to the bottom wells and incubated for 24 hours at 37°C in normoxia or hypoxia. Migration assay was also performed with the transfected cells. Cells that did not migrate were gently discarded from the top, the migration chamber plate then placed onto a new feeder tray containing pre-warmed cell detachment solution, and incubated for 30 min at 37°C. Cells that migrated were lysed using a lysis buffer containing the patented CyQuant green-fluorescent dye (Molecular Probes), and fluorescence determined at 480/520 nm. Differences among control and treated groups were expressed as percent of control (assigned 100%) and shown in bar diagrams with means ± standard error.

**Data analysis.** Student’s t test for unpaired observations was used to compare the mean values of two groups. Comparison of mean values of more than two groups was made with one-way
ANOVA test with Student-Newman-Keuls test for post hoc testing of multiple comparisons. Statistical significance was accepted when the \( p \) value (two tailed) was less than 0.05.

RESULTS

**Hypoxia increases cell adhesion to matrix proteins:** The effect of hypoxia on pulmonary artery smooth muscle cell adhesion was studied after 24 hours of treatment (Figure 1A). We explored cell adhesion to different matrix proteins (collagen I, collagen II, collagen IV, fibronectin, laminin and vitronectin) and found that cell adhesion to the four matrix proteins increased by 25-50% after 24 hours of hypoxia. The increase noted was statistically significant for collagen I, collagen IV, and tenacin with a trend towards significance for cell adhesion to fibronectin and laminin. The role of PKG in the modulation of cell adhesion properties in hypoxia was studied by treating cells with the PKG1 specific inhibitor, DT-3, for 24 hours under normoxic conditions. PKG inhibition resulted in increased cell adhesion, findings similar to that of cells treated with hypoxia. The effect of PKG1 inhibition with DT-3 in hypoxia was examined by monitoring adhesion properties of cells exposed to hypoxia in the presence of DT-3 (Figure 1B). We found that, in hypoxia, DT-3 leads to a significant decrease in adhesion, below that observed in normoxia and speculate that this may be due to additive effects of hypoxia and DT-3 on PKG activity or perhaps due to non-specific effects.

**Effect of hypoxia on integrin binding:** Since extracellular matrix protein adhesion is mediated by integrins, we examined the effects of hypoxia treatment on \( \alpha \)- (A) and \( \beta \)-integrin (B) binding in FPASMC (Figure 2). FPASMCs were exposed to normoxia, hypoxia or DT-3 in normoxia for 24 hours. Under normoxia, significant binding to \( \alpha_1, \alpha_2, \alpha_V\beta_3, \beta_1 \) and, to a lesser extent, to \( \alpha_4, \alpha_5\beta_1 \) was observed. Treatment with hypoxia or with DT-3 increased adhesion to these integrins, with the most significant effect being on \( \alpha_4, \beta_1 \) and \( \alpha_5\beta_1 \).

**Hypoxia decreases PKG expression and kinase activity:** The constitutive PKG kinase activity, in the absence of exogenous cGMP, in FPASMC under normoxia or hypoxia was significantly lower than PKG kinase activity in the presence of exogenous cGMP (Figure 3A). This observation is consistent with the ability of cGMP to bind and activate PKG (14). Constitutive and cGMP-stimulated PKG kinase activities were reduced by treatment with DT-3 in normoxia or hypoxia compared with control, while exposure to hypoxia alone inhibited the cGMP-stimulated kinase activity. Immunoblot analyses indicate that, under similar conditions, PKG protein expression is decreased by exposure to hypoxia or DT-3 (Figure 3B).

**PKG inhibition with expression of dominant negative PKG construct mimics the effect of hypoxia on cell adhesion:** To further study the role of PKG in modulating cell adhesion properties, we transiently transfected FPASMCs with plasmids encoding full-length PKG1\( \alpha \) tagged with green fluorescent protein (PKG-GFP), a dominant negative construct consisting of a truncation encoding the regulatory domain of PKG1\( \alpha \) (PKG G1\( \alpha \)R-GFP) or vector alone (pEGFP-N1). Figure 4A shows that there is a significant increase in cell adhesion to fibronectin in PKG G1\( \alpha \)R-GFP-transfected cells when compared to mock (pEGFP-N1 vector)-transected or PKG-GFP-transfected cells. Hypoxia induced a significant increase in adhesion in cells transfected with vector alone, comparable to that of cells treated with hypoxia and cells treated with DT-3 in the
presence of normoxia (Figure 1A), whereas PKG overexpression blunted this effect. A slight but insignificant increase in adhesion was observed upon exposure to hypoxia of cells expressing the dominant negative construct (PKG G1αR-GFP). Transfection efficiency in these cells was confirmed by analysis of PKG protein expression (Figure 4B) and kinase activity (Figure 4C). Expression of wild type PKG induces a significant increase in constitutive and cGMP-stimulated kinase activities, while the dominant negative construct inhibits both. The effect of PKG expression on vasodilator-stimulated phosphoprotein (VASP) phosphorylation levels was examined by immunoblot detection of VASP Ser239. PKG mediated phosphorylation of VASP, a ubiquitously expressed endogenous substrate for PKG and biomarker for activation of the cGMP/PKG pathway, was markedly elevated in cells expressing PKG (Figure 4D).

**Hypoxia and PKG inhibition with DT-3 or by expression of a dominant negative construct increase cell migration:** The effect of hypoxia on cell migration was studied using the wound-healing model (Figure 5A). There was a significant increase in cell migration at 24 hours of hypoxia. To confirm the findings seen using the wound-healing model, we studied cell migration using the Boyden chamber method. We found a statistically significant increase in cell migration in cells exposed to hypoxia (Figure 5B). There was a statistically significant increase in cell migration when cells were treated with DT-3 (Figure 5), as was seen in cells treated with hypoxia. The role of PKG in cell migration was further evaluated using FPASMC transiently transected with plasmids encoding full-length PKG1α (PKG-GFP) or a dominant negative PKG1α construct (PKG G1αR-GFP) or vector alone. Figure 5D shows that there is a small, but significant decrease in cell migration in PKG1α expressing cells, while PKG G1αR-GFP transfection induced an increase in cell migration compared to vector only transfected cells. In all cases, hypoxia induced increased cell migration, though the effect observed with wt PKG-expressing cells was blunted. These findings are comparable to that of cells treated with hypoxia and cells treated with DT-3 in the presence of normoxia.

**Modulation of cofilin phosphorylation by PKG.** Integrin-mediated actin reorganization and focal adhesion formation, critical components of cell adhesion and migration, are regulated by actin-binding proteins such as cofilin (23). The activity of cofilin is reversibly regulated by phosphorylation and dephosphorylation at Ser-3, with the phosphorylated form being inactive. To assess the possible role of PKG in modulating cofilin activation, we determined cofilin expression and phospho-cofilin(Ser3) levels in FPASMC transiently transfected with vector alone (pEGFP-N1, mock), PKG-GFP or PKG 1αR-GFP (dominant negative) constructs (Figure 6A). Cofilin expression was unaltered, while cofilin phosphorylation changed depending on the expression and activity level of PKG. Cofilin phosphorylation decreased in PKG-GFP expressing cells and increased in PKG G1αR-GFP expressing cells. Treatment with cGMP, in all three cases, led to a slight decrease in cofilin phosphorylation. The role of hypoxia in modulating cofilin phosphorylation was further assessed by exposing cells to normoxia or hypoxia under conditions of PKG activation or inhibition (Figure 6B). Upon exposure to hypoxia, cellular p-cofilin(Ser3) levels were elevated, and this increase was eliminated when PKG was activated by the addition of cGMP. The inhibition of PKG with DT-3 led to increased cofilin phosphorylation both in normoxia and hypoxia. These data suggest that PKG may play a role in the modulation of upstream signaling pathways that phosphorylate and dephosphorylate cofilin.
DISCUSSION

This is the first study to link cGMP-dependent protein kinase (PKG) to hypoxia-induced changes in adhesion and migration properties of pulmonary artery smooth muscle cells. We have previously reported that hypoxia downregulates PKG (9; 28), and that this downregulation is associated with altered SMC phenotype characterized by changes in the expression of SMC marker proteins (28). Here, we demonstrate that cell adhesion to matrix proteins increases when PKG is inhibited, findings that mimic the effects of hypoxia. We also show that cell migration is increased both with hypoxia and with PKG inhibition in normoxia. These findings suggest that the downregulation of PKG with hypoxia may be the mechanism by which hypoxia alters cell adhesion and migration.

cGMP-dependent protein kinase (PKG) plays a key role in nitric oxide-cGMP mediated pulmonary vascular relaxation (8). We chose to study PKG since we have shown previously that PKG activity is decreased in hypoxia and that it is involved in smooth muscle cell phenotype modulation (28). PKG expression has also been shown to be decreased in pulmonary artery smooth muscle cells cultured from an ovine fetal model of chronic intrauterine pulmonary hypertension (19). Murphy-Ullrich et al (16) have shown that PKG is necessary to mediate focal adhesion disassembly triggered by the counter-adhesive extracellular matrix protein thrombospondin and tenascin in rat aortic smooth muscle cells. The present study is the first to establish the role of PKG in hypoxia-induced changes in pulmonary vascular smooth muscle cell adhesion and cell migration. Our data show that inhibition of PKG leads to an increase in cell adhesion and migration. This would suggest that the decrease in PKG activity associated with hypoxia may be the mechanism by which hypoxia alters cell adhesion and migration. Therefore, we can propose that PKG may be a regulator of cell adhesion and migration in hypoxia.

Exposure to hypoxia can lead to pulmonary hypertension and is accompanied by pulmonary vascular cell proliferation and vascular remodeling (18). Environmental stressors such as hypoxia induce multiple effects including phenotypic change of vascular smooth muscle cells from the quiescent contractile state to the active synthetic state, demonstrating increased motility and proliferation (27). Many studies of cell adhesion and migration in hypoxia have been limited to circulating blood cells, where an increase in adhesion has been shown in response to hypoxia (6; 22; 26). Corley et al 2005 (5), however reported a decrease in adhesion of vascular smooth muscle cells from bovine coronary arteries when treated with cobalt chloride treatment, an agent which simulates hypoxia. In our study, we show a significant increase (25-50%) in vascular smooth muscle cell adhesion in response to hypoxia. The differential response may be attributed to the different source of cell type.

The regulation of vascular smooth muscle cell adhesion and migration in hypoxia is poorly understood, however several groups have studied different pathways. There have been reports of specific proteins involved in promoting cell migration in hypoxia in different cell types, such as hypoxia-induced mitogenic factor in endothelial cells (24) and EGFR in adenocarcinoma cells (25). There have been reports showing the regulation of migration of vascular smooth muscle cells through the mitogen activated protein kinase ERK1/2 (2; 14). Corley 2005 et al (5) reported the role of hypoxia-inducible factor in regulating vascular smooth muscle cell adhesion and migration. However, they report a decrease in cell adhesion in vascular smooth muscle
cells when treated with cobalt chloride. Li et al (11) found an increase in the expression of the novel adhesion molecule periostin and osteopontin expression in hypoxia and demonstrated the role of the PI3K and Ras signaling pathways. These observed changes in adhesion and migration are important in understanding the steps leading to increased proliferation seen in hypoxia. The current study is the first to link PKG to the hypoxia-induced changes in vascular smooth muscle cell adhesion and migration.

Pulmonary artery remodeling is a complex pathological process involving all three layers of the vascular wall, including smooth muscle cell proliferation (21). Cell adhesion and migration are important factors involved in the regulation of cell proliferation, and alterations in cell adhesion and migration properties are necessary for cells to proliferate. Migrating cells extend protrusions in the direction of migration and are stabilized by adhering to the extracellular matrix (20). These adhesion sites provide traction for the migrating cells as the cell moves forward, while they are disassembled at the cell rear, allowing the cell to detach. Protein kinases have been shown to be central in the regulation of adhesion stability and turnover (20). Since hypoxia leads to pulmonary artery smooth muscle cell proliferation, as evidenced by vascular remodeling, we decided to focus our investigation on the effects of hypoxia on cell adhesion and migration and the role of PKG. Others have shown the role of kinases in the regulation of migration in normoxia. Several groups have demonstrated that PKCδ regulates vascular smooth muscle cell adhesion and migration (10; 14). Here, we show that hypoxia increases cell adhesion to matrix proteins and migration in PASMC and that PKG seems to be mediating this process in hypoxia. Our findings agree with previous data suggesting that vascular smooth muscle cell adhesion to matrix proteins is increased in hypoxia (2). The discrepancy in our finding that DT-3 can augment VSMC adhesion and migration under hypoxia, while inhibiting hypoxic VSMC adhesion and migration suggests that DT3 may have non-specific effects in hypoxia. As we have reported previously (17), hypoxia induces increased generation of cellular reactive oxygen and nitrogen species. Under such conditions, various target proteins, including kinases involved in the regulation of VSMC adhesion and migration could be modified by ROS/RNS and their structures altered, possibly becoming more susceptible to inhibition by DT-3 at the concentration used under our experimental conditions.

The actin cytoskeleton is a dynamic structure that plays an integral role in regulating cellular function and architectural integrity. The dynamics of polymerization / depolymerization of actin filaments, essential for cell movement, adhesion, and division (23), are coordinately regulated by a group of actin-binding proteins including cofilin (4). Cofilin, in turn, is negatively regulated by Ser3 phosphorylation. The current study demonstrates, for the first time, that PKG is a critical modulator of cofilin (Ser3) phosphorylation and thus actin cytoskeletal dynamics in VSMC.

In summary, we report that hypoxia increases both vascular smooth muscle cell adhesion and migration, and the response seen with hypoxia is mediated by PKG, as hypoxia downregulates PKG. Inhibitors to PKG also increase cell-extracellular matrix protein interactions and cell migration, results that are comparable to the effects of hypoxia. Our data suggest that PKG plays a critical role in regulating vascular smooth muscle cell adhesion and migration in hypoxia in part through the regulation of cofilin (Ser3) phosphorylation.
Grants

This study was supported by National Heart, Lung, and Blood Institute Grants R01 HL-59435-05, R01 HL-075187, and R01/MIRS HL-75187-01.
REFERENCES


FIGURE LEGENDS

Figure 1. (A) Hypoxia-induced increased adhesion of FPASMC to extracellular matrix proteins and effect of pharmacological inhibition of cGMP-dependent protein kinase (PKG) by DT-3. Col I, col II, col IV, FN, LN, TN and VN correspond to collagen I, collagen II, collagen IV, fibronectin, laminin and vitronectin coated wells, respectively. FPASMC were exposed to normoxia, hypoxia or normoxia in the presence of DT-3 (3 μM) for 24 h, harvested and incubated for 1h at 37ºC in wells coated with extracellular matrix proteins. Values are means ± SE (n=4). *Significantly different from respective values observed in control (P < 0.05). (B) Effect of DT-3 on adhesion of FPASMC exposed to hypoxia to fibronectin. Cells were incubated in normoxia or hypoxia in the presence or absence of DT-3 for 24 h. Values are means ± SE (n=4). *Significantly different from normoxia (-DT-3) (P < 0.05).

Figure 2. (A) α-Integrin and (B) β-integrin mediated binding profile of FPASMC exposed to normoxia, hypoxia or normoxia in the presence of PKG inhibitor DT-3 (3μM) for 24 h, harvested and incubated for 1h at 37ºC in wells coated with anti-alpha or anti-beta integrin monoclonal antibodies. Values are means ± SE (n=3). *Significantly different from respective values observed in control (P < 0.05).

Figure 3. Downregulation of PKG kinase activity (A) and protein expression (B) in FPASMC after exposure to normoxia or hypoxia for 24 h in the presence or absence of 3 μM DT-3. (–) cGMP, without cGMP; (+) cGMP, with 3 × 10⁻⁶ M cGMP. Values are means ± SE (n = 4). *Significantly different from respective (–) cGMP values (P < 0.05). #Significantly different from normoxia (+) cGMP value (P < 0.05). B: PKG and actin expression in cell extracts of FPASMC exposed to 24 h normoxia or hypoxia in the presence or absence of DT-3. Top: representative blots of PKG and actin expression in homogenates exposed to normoxia (control, lane 1), normoxia + DT-3 (lane 2), hypoxia (lane 3) or hypoxia + DT-3 (lane 4). Bottom: densitometric quantification of PKG protein normalized to actin. AU, arbitrary units. Values are means ± SE (n = 4). *Significantly different from normoxia (-DT-3) (P < 0.05).

Figure 4: Increased cell adhesion to fibronectin by PKG inhibition with transient transfection of FPASMC with a GFP-tagged dominant negative PKG construct. A. FPASMC transfected with pEGFP-N1 vector (mock), wt PKG (PKG-GFP) or PKG 1αR-GFP (dominant negative) were exposed to 24h normoxia or hypoxia, harvested and allowed to adhere to fibronectin-coated wells for 1h at 37ºC. Values are means ± SE (n=3). *Significantly different from mock, normoxia value (P < 0.05). Immunoblot detection of PKG expression (B) using anti-PKG antibody and measurement of kinase activity (C) in the presence or absence of 3 μM cGMP in FPASMC transiently transfected with vector alone (mock) wt PKG (PKG-GFP) and a dominant negative PKG construct (G1αR-GFP). Because of the similarity in size of GFP (~30kDa) to the deleted portion of PKG, the molecular weight of the fusion protein is 85kDa. *Significantly different from respective (–)cGMP values (P < 0.05). #Significantly different from normoxia (+)cGMP value (P < 0.05). (D) Expression of endogenous PKG substrate, vasodilator-stimulated phosphoprotein (VASP), and immunodetection of phospho-VASP (Ser239) in transfected cells.
Figure 5: Downregulation of PKG in hypoxia or inhibition of PKG in normoxia with a dominant negative PKG construct (G1αR-GFP) or PKG1 inhibitor DT-3 (3μM) lead to increased FPASMC migration. A. Hypoxia-induced increase in migration of FPASMC using wound healing model. Scratch on cell monolayer (0 hr) and migration at 24 hours normoxia and hypoxia. B. Migration was assessed at 24 hours by counting the number of cells that migrated across the scratch, normalized to scratch area and presented as a percentage, with normoxia set at 100%. Values are means ± SE (n=4). C. Boyden chamber migration assay of FPASMC treated with normoxia, hypoxia, or normoxia in the presence or absence of PKG1 inhibitor (DT3, 3μM). Migration is presented as a percentage, with normoxia (–DT3) set at 100%. Values are means ± SE (n=3). *Significantly different from normoxia (–DT3) value (P < 0.05). D. Boyden chamber migration assay of FPASMC transfected with vector alone (pEGFP-N1, mock), PKG-GFP or PKG1αR-GFP (dominant negative) constructs. Migration is presented as a percentage, with vector set at 100%. Values are means ± SE (n=3). *Significantly different from respective normoxia values (P < 0.05). #Significantly different from mock, normoxia value (P < 0.05).

Figure 6. Modulation of cofilin phosphorylation by PKG. A. Cofilin expression and phosphocofilin(Ser3) levels were determined in cell extracts prepared from FPASMC transfected with vector alone (pEGFP-N1, mock), PKG-GFP or PKG1αR-GFP (dominant negative) constructs and incubated in the presence or absence of cGMP (3μM, 10min, 37°C). Top: representative blots of phospho-cofilin(Ser3) and cofilin expression. Bottom: Densitometric quantification of phospho-cofilin levels normalized to cofilin expression in each sample. AU, arbitrary units. Values are means ± SE (n = 4). *Significantly different from respective –cGMP values (P < 0.05). #Significantly different from PKG-GFP, –cGMP value (P < 0.05). †Significantly different from PKG-GFP –cGMP and +cGMP values (P < 0.05). B. Cofilin expression and phospho-cofilin(Ser3) levels were determined in FPASMC exposed to normoxia (lanes 1, 3 and 5) or hypoxia (lanes 2, 4 and 6) in the presence of cGMP (lanes 3 and 4) or DT-3 (lanes 5 and 6). FPASMC were exposed to normoxia, hypoxia or normoxia in the presence or absence of DT-3 (3μM) for 24 h. cGMP (3μM, 10min, 37°C) was added to cells 10 min prior to the end of a 24 h incubation period.
Figure 2A.

Figure 2B.
Figure 3A.

![PKG Kinase Activity Graph]

Figure 3B.

![PKG and Actin Western Blots]

Band Intensity (A.U.)

- Normoxia
- Hypoxia

(-) DT3  (+) DT3

* indicates a significant difference compared to Normoxia.

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Figure 4A. Adhesion (OD 570 nm)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normoxia</th>
<th>Hypoxia</th>
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<tbody>
<tr>
<td>MOCK</td>
<td>1.0 ± 0.1</td>
<td>0.8 ± 0.1</td>
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<tr>
<td>PKG-GFP</td>
<td>0.6 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>G1αR-GFP</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

Figure 4B. Western Blot
- PKG
- GFP
- Actin

Figure 4C. PKG Kinase Activity (pmol/min/mg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(-) cGMP</th>
<th>(+) cGMP</th>
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</thead>
<tbody>
<tr>
<td>MOCK</td>
<td>150 ± 10</td>
<td>150 ± 10</td>
</tr>
<tr>
<td>PKG-GFP</td>
<td>750 ± 50</td>
<td>750 ± 50</td>
</tr>
<tr>
<td>G1αR-GFP</td>
<td>300 ± 20</td>
<td>300 ± 20</td>
</tr>
</tbody>
</table>

Figure 4D. Western Blot
- p-VASP (Ser239)
- VASP
Figure 5A

Figure 5B

Migration (% of Normoxia)

Normoxia  Hypoxia

*
Figure 5C

![Graph showing migration (\% Control) under normoxia and hypoxia conditions.](image)

Figure 5D

![Graph showing migration (\% Control) under different treatments.](image)
Figure 6A

![Blot with bands for p-cofilin(Ser3) and cofillin under different conditions](image)

- **cGMP**
  - **Mock**
  - **PKG-GFP**
  - **G1αR-GFP**

Figure 6B

![Blot with bands for p-cofilin(Ser3) and cofillin under different conditions](image)

- **Control**
  - **+cGMP**
  - **+DT3**

Band Intensity (A.U.)

<table>
<thead>
<tr>
<th></th>
<th>MOCK</th>
<th>PKG-GFP</th>
<th>G1αR-GFP</th>
</tr>
</thead>
<tbody>
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<td><img src="image" alt="Bar" /></td>
<td><img src="image" alt="Bar" /></td>
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<tr>
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<td><img src="image" alt="Bar" /></td>
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</tr>
</tbody>
</table>

Legend:
- *: p < 0.05
- #: p < 0.01
- +: p < 0.001