Research Article

Hypoxia modulates the expression of leucine zipper-positive MYPT1 and its interaction with protein kinase G and Rho kinases in pulmonary arterial smooth muscle cells

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ABSTRACT

We have shown previously that acute hypoxia downregulates protein kinase G (PKG) expression and activity in ovine fetal pulmonary vessels and pulmonary arterial smooth muscle cells (SMC). Here, we report that acute hypoxia also reduces the expression of leucinezipper-positive MYPT1 (LZ^+ MYPT1), a subunit of myosin light chain (MLC) phosphatase, in ovine fetal pulmonary arterial SMC. We found that in hypoxia, there is greater interaction between LZ^+MYPT1 and RhoA and Rho kinase 1 (ROCK1)/Rho kinase 2 (ROCK2) and decreased interaction between LZ^+MYPT1 and PKG, resulting in increased MLC_{20} phosphorylation, a higher $pMLC_{20}/MLC_{20}$ ratio and SMC contraction. In normoxic SMC PKG overexpression,LZ^+MYPT1 expression is upregulated while PKG knockdown had an opposite effect. LZ^+MYPT1 overexpression enhanced the interaction between PKG and LZ^+MYPT1. Overexpression of a mutant LZ^-MYPT1 isoform in SMC mimicked the effects of acute hypoxia and decreased $pMLC_{20}/MLC_{20}$ ratio. Collectively, our data suggest that hypoxia downregulates LZ^+MYPT1 expression by suppressing PKG levels, reduces the interaction of LZ^+MYPT1 with PKG and promotes LZ^+MYPT1 interaction with RhoA or ROCK1/ROCK2, thereby promoting pulmonary arterial SMC contraction.

Key Words: hypoxia-induced pulmonary hypertension, signal transduction, cGMP, pulmonary vasoconstriction

INTRODUCTION

Acute hypoxia leads to a rapid reversible physiological response of pulmonary vasoconstriction (reviewed in[1]). Myosin light chain (MLC) phosphorylation is a critical step that leads to activation of myosin ATPase and subsequent smooth muscle cell (SMC) contraction. Myosin light chain 20 (MLC_{20}) is phosphorylated by MLC kinase (MLCK) and de-phosphorylated by MLC phosphatase (MLCP). Although a recent study has shown that MAP kinase phosphorylates MLC independent of MLCK in urinary bladder SMC,[2] MLCK is still considered to be the main enzyme responsible for phosphorylation of MLC_{20} and MLC phosphatase is the main enzyme involved in regulation of MLC_{20}dephosphorylation and vasodilatation.[3,4] In general, the contractile state of the SMC reflects the ratio of activities of MLCK/MLCP and $pMLC_{20}/MLC_{20}$.

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MLCP is a hetero-trimeric protein composed of a catalytic subunit PP1Cδ (*see* References 5 and 6 for review), a regulatory subunit also known as myosin phosphatase targeting unit (MYPT) and a 20 kD protein of unknown function. Regulatory subunits of the MYPT family are the central targeting proteins thatinteract with a number of other signaling molecules such as protein kinase G (PKG), Rho kinase 1 (ROCK1) and Rho kinase 2 (ROCK2) (reviewed in Reference 7). There are fourdifferent types of MYPT1 monomers produced endogenously by splicing, i.e. MYPT1 with or without central insert (CI), and both these forms are further spliced with (LZ^+MYPT1) or

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without (LZ^-MYPT1) LZ, a leucine zipper at the c-terminal. The specificity of MLCP is primarily determined by the interaction of PP1cä with the targeting protein MYPT.[8,9] It has been reported that the sensitivity of smooth muscle to cGMP-induced relaxation correlates with the relative expression of LZ^+MYPT1 and LZ^-MYPT1 isoforms, as overexpression of LZ^+ MYPT1 or LZ^- MYPT1 isoforms in cultured SMC modulates cGMP-mediated MLC_{20} dephosphorylation.[10-12] Smooth muscle contractility is modulated by the ratio of phosphorylated MLC_{20} to unphosphorylated MLC_{20} ($pMLC_{20}/MLC_{20}$), and this ratio is regulated by the relative activities of MLCK/MLCP.[3,4]

We tested the hypothesis that hypoxia regulates the level of LZ^+MYPT1 and LZ^-MYPT1 isoforms, and thereby SMC contractility, in cultured ovine fetal pulmonary arterial SMC (FPASMC). We report that hypoxia downregulates the expression of LZ^+MYPT1, a predominant isoform in vascular smooth muscle in a PKG-dependent manner, reduces the interaction between LZ^+MYPT1 andPKG and promotes its interactions with ROCK1/ROCK2, resulting in increased MLC phosphorylation and SMC contraction.

MATERIALS AND METHODS

Reagents

Trolox [(+)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid], N-acetyl cysteine (NAC) and all other chemicals (unless otherwise specified) were obtained from Sigma-Aldrich (St. Louis, Mo., USA). 8-Bromo-cGMP (8-Br-cGMP) was purchased from Axxora LLC/Biolog Life Science Institute (San Diego, Calif., USA).

Animals

Pregnant ewes carrying single or twin fetuses (140 days of gestation; term being 147 days, either sex) were obtained from Nebeker Ranch in Lancaster, California (altitude 300 m.; arterial PO_2 (PaO_2): 102 +2 Torr). After the fetuses were delivered, each ewe was euthanized with T-61 euthanasia solution (Hoechst-Roussel, Somerville, NJ, USA). All procedures and protocols used in the present study were approved by the Animal Research Committees of Loma Linda University, Los Angeles Biomedical Research Institute at Harbor-UCLA and by the University of Illinois at Chicago.

Tissue preparation and cell culture

Fourth-generation pulmonary arteries (outside diameter: 1.5–2.5 mm) were dissected free of parenchyma and kept in ice-cold modified Krebs-Ringer bicarbonate buffer (composition in mM: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 11.1 glucose) and primary ovine FPASMC were isolated from pulmonary arteries as described earlier.[13] Cells were maintained in DMEM containing 10% heat-inactivated fetal bovine serumand antibiotics (Invitrogen, Carlsbad, Calif., USA). Primary ovine FPASMC were confirmed to beSMCs by their typical "hill and valley" morphology and by α-smooth muscle actin immunofluorescent staining. Contamination with endothelial cells is ruled out by negative immunofluorescent staining with an anti-von Willebrand factor VIII antibody. All experiments were performed with cells at passages 4–6.

Hypoxia exposure

Subconfluent FPASMC were serum-starved (0.5% serum) for 16 h and then exposed to 4 h of hypoxia or normoxia at 37°C, as previously described.[13] Briefly, cells were placed in a specialized hypoxia chamber into which a gas mixture of 0% O_2, 5% CO_2 and balance nitrogen was flowed in at a rate sufficient to keep O_2 concentration within the chamber at 3%. The concentration of O_2 within the chamber was constantly monitored and controlled with an O_2 controller (Model ProOxC, Biospherix, Lacona, NY, USA).[13] The PO_2 in the cell media during hypoxia was 30–40 Torr and during normoxia was ~100 Torr. For normoxia experiments, cells were incubated in a humidified incubator with a constant supply of 5% CO_2 at 37°C.

siRNA transfection

We used PKG-specific small interfering ribonucleic acid (siRNA) to knockdown PKG expression in FPASMC.[13]A nonsilencing oligonucleotide sequence (nonsilencingsiRNA) that does not recognize any known homology to mammalian genes was used as a negative control (Qiagen, Valencia, Calif., USA cat. no. 1022563). FPASMC were transfected with siRNAs using Lipofectamine 2000 reagent (Invitrogen). Fresh medium was added after 6 h of transfection and the cells were further cultured for 24 hbefore exposing them to hypoxia or normoxia for 4 h. PKG1α expression level and activity was determined as described earlier.[13] FPASMC were treated with 2×10^{-4}M Trolox (an $ONOO^-$ scavenger) or 1×10^{-3}M NAC for 30 min before exposure to 4 h hypoxia.

Transient transfections

To overexpress PKG1α, we used the Lenti-X overexpression system (Clontech, Mountain View, Calif., USA) as recommended by the manufacturer. In brief, Lent-X vector containing a full-length PKG1α tagged with green fluorescent protein (GFP) was co-transfected along with a Lenti-X HT Packaging Mix into 293 T cell line using Lipofectamine 2000. Lentiviral supernatant produced by the transfected packaging cells was then used to infect FPASMC along with polybrene (4μg/mL). FPASMC overexpressing PKG1α were selected by Puromycin (1.5 μg/mL). Lentiviral negative control cells were included in all the experiments. After 24 h of transfection,

cells were serum-starved overnight before exposure to hypoxia or normoxia for 4 h.

A full-length MYPT1 ($^{+}$LZ) construct with all the functional domains in MYPT1 and a MYPT1 ($^{-}$LZ) construct lacking the LZ domain, gifts from F.V. Brozowich,[14] were purified and their sequence verified before use in experiments. Cells weretransfected with MYPT1^{+}LZ and MYPT^{-}LZconstructs using lipofectamine 2000 (Invitrogen) and subsequently exposed to normoxia. Empty vector, pcDNA3.1, was transfected as a negative control. Transfection efficiency was analyzed by immunoblot analysis using appropriate antibodies.

Western blot analysis

After each experimental exposure, FPASMC were harvested in RIPA buffer (20 mMTris-HCl–pH 7.5, 150 mMNaCl, 1 mM EDTA, 1 mM EGTA, 1% IGEPAL, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich) and protein concentration was determined using the Bradford protein assay kit (Bio-rad, Hercules, Calif., USA). Equal amounts of total protein (10–50 μg) from cell lysates were subjected to sodium dodecyl sulfate—polyacrylamide gel electrophoresisand blotted onto nitrocellulose membrane and probed with primary antibodies according to the manufacturer's instructions. Horseradish peroxidase (GE Life Sciences, Piscataway, NJ, USA) was used as secondary antibody at concentrations from 1:2,000 to 1:20,000. Immunoreactive bands were detected using SuperSignal West Pico Chemi-luminescent Substrate (Pierce , Rockford, Ill). The relative intensities of immunoreactive bands were quantified by densitometry using β-actin band as a reference. The primary antibodies used for this study include anti-MYPT1, anti-pMYPT1 (Ser^{695}) and anti-MLC_{20} (Santa Cruz Biotech, Santa Cruz, Calif.); anti-$pMLC_{20}$ (Ser^{18}/Ser^{19}) from Cell Signaling, Danvers, Mass., USA; anti-PKG1α and anti-β-actin antibodies from Calbiochem, San Diego, CA, USA; and MYPT1 monoclonal antibody (BD) and LZ^{+}MYPT1 antibody (custom antibody from Affinity Bioreagent, Rockford, Ill., USA). The amount of PKG-mediated MLCP phosphorylation was determined by measuring phosphorylation of MYPT1 at Ser^{695} using pMYPT1Ser^{695} antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). Also, phosphorylation of MLC at Thr^{18}/Ser^{19} and MLC_{20} was evaluated by immunoblotting with the respective antibodies (Santa Cruz Biotechnology).

Immunoprecipitation

Total proteins from FPAMSC were extracted in RIPA buffer after exposure to acute hypoxia or normoxia for 4 h, as described earlier. One microgram of polyclonal anti-RhoA, ROCK 1, ROCK2 or PKG1α antibody was prebound with Ultralink immobilized protein A/G (Pierce) by gentle shaking for 1 h at room temperature. The prebound primary antibody and protein A/G agarose complex was then added to each sample (200 μg protein) and incubated at 4°C overnight. The agarose beads were washed in tris buffered saline (TBS) three-times and boiled in 1x sample buffer (Bio-rad). Western blot analysis was performed as described above.

Statistical analysis

Statistical analysis of the data was performed using a standard two-sample Student's t-test assuming unequal variances of the two data sets and one-way ANOVA for the comparison of multiple experimental groups. Statistical significance was determined using a two-tailed distribution and was set at the 5% level ($P<0.05$).

RESULTS

Hypoxia downregulates PKG and LZ^{+}MYPT1 expression in FPASMC

Cells at 75% confluence were exposed to hypoxia or normoxia for 4 h, cell lysates prepared and separated on acrylamide gels. Membranes were probed with anti-PKG, anti-LZ^{+}MYPT1 or anti-â-actin antibodies. Bands were quantified by densitometry using â-actin as a reference. As shown in Figure 1, hypoxic exposure significantly decreased (by 20–35%) the expression levels of PKG (Fig. 1 a and b) and LZ^{+}MYPT1 (Fig. 1 c and d) in FPASMC.

PKG Knockdown mimics the effects of acute hypoxia on LZ^{+}MYPT1 expression in FPASMC

Next, we determined whether lower expression levels of PKG would mimic the effects of acute hypoxia on LZ+MYPT1 expression. FPASMC were transfected with siRNA specific for PKG (si-PKG) or nonsilencingsiRNA that does not recognize any known homology to mammalian genes. PKG and LZ^{+}MYPT1 expression was analyzed by Western blot analysis. There was a 45–60% decrease in PKG expression in siPKG-transfected cells compared with control cells (Fig. 2a and b). There was no significant change in the expression level of PKG and LZ+MYPT1 between mock-transfected FPASMC and nonsilencingsiRNA-transfected control cells (Fig. 2a–d). PKG knockdown caused decreased LZ^{+}MYPT1 expression (Fig. 2c and d). This result suggests that hypoxia downregulates LZ^{+}MYPT1 expression by reducing PKG expression.

Overexpression of PKG abrogates the effect of acute hypoxia on LZ^{+}MYPT1 expression

To determine whether overexpression of PKG can abrogate the effect of acute hypoxia on LZ^{+}MYPT1 expression, a plasmid encoding a full-length PKG1α (PKG-GFP) tagged with a GFP was transfected into FPASMC exposed to

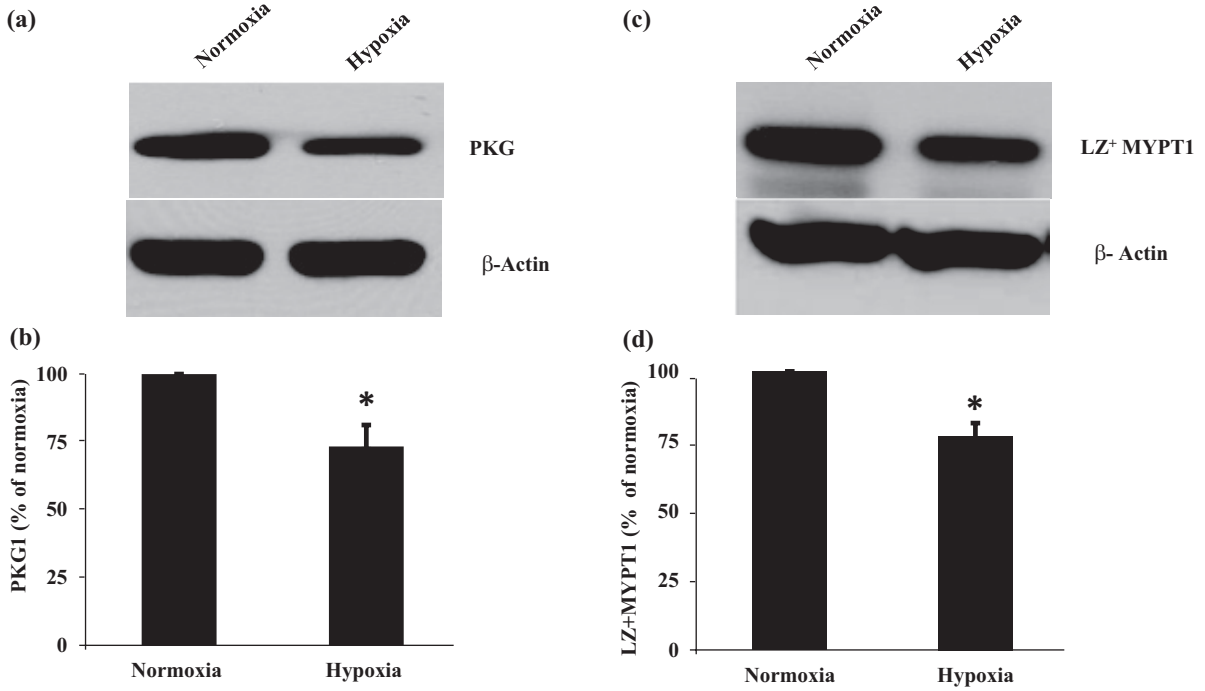

Figure 1: Effect of hypoxia on protein kinase G (PKG) and LZ+MYPT1 expression.Fetal pulmonary arterial smooth muscle cells were exposed to acute hypoxia (4 h) and cell lysates were prepared and probed with anti-PKG1á, anti-LZ+MYPT1 and â-actin antibodies. (a and c) Western blot analysis of PKG and LZ+MYPT1, respectively.(b and d) Quantification of PKG and LZ+MYPT1 expression. Bands were quantified and normalized to that of â-actin bands. The values are shown as percent of normoxia control after normalizing the data to respective â-actin values. Data represent means±SE, from at leastthree3 independent experiments. *P<0.05 compared with normoxia.

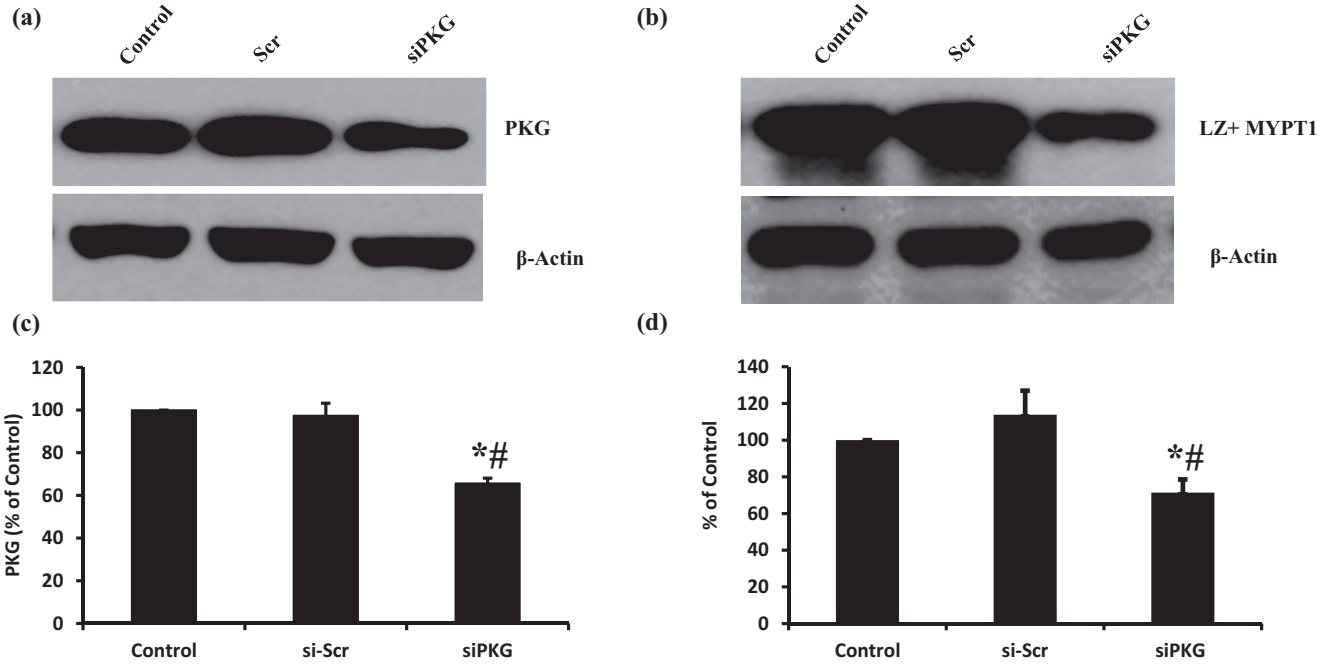

Figure 2: The effect of protein kinase G (PKG) knockdown on LZ+MYPT1 expression. Fetal pulmonary arterial smooth muscle cellsFPASMC were transfected with siRNA specific for PKG or non-silencings iRNA, as a negative control. Cell lysates were isolated and probed with antibodies specific for PKG, LZ+MYPT1, and ↓-actin. (a) Representative Western blot analysis of PKG expression. (b) Quantification of PKG expression. Bands were quantified and normalized to that of ↓-actin bands. (c and d) Analysis of LZ+MYPT1 expression in PKG-–siRNA siRNA-transfected cells. Samples as in panel A, were probed with antibodies specific for LZ+MYPT1 and ↓-actin. (d) Densitometry quantification of LZ+MYPT1 expression. Data represent means±SE from at least three to six3-6 independent experiments. *P<0.05 compared with the respective control (un-transfected cells).

normoxia or hypoxia for 4 h and the expression of PKG1á and LZ+MYPT1 was analyzed by immunoblot analysis. Overexpression of PKG1α (Fig. 3 a and b) significantly increased LZ+MYPT1 expression in normoxic cells and also blocked the inhibitory effects of hypoxia on LZ+MYPT1 expression (Fig. 3c and d). These data further support our contention that hypoxia by reducing PKG1á expression suppresses LZ+MYPT1 levels in FPASMC.

Reactive oxygen species, by reducing PKG expression, decrease LZ+MYPT1 levels in hypoxia

Reactive oxygen species (ROS) generated in hypoxia[23,28-30] have been shown to play a role in the downregulation of PKG expression.[15] To test whether ROS are directly involved in the downregulation of LZ+MYPT1 expression, FPASMC were pretreated with ROS scavengers, N-acetyl cysteine (NAC) or trolox (scavenger of peroxynitrite) before exposing them to hypoxia. There was no change in the expression level of PKG or LZ+MYPT1 in cells preincubated with trolox or NAC under normoxia (Fig. 4). However, trolox or NAC blocked the downregulation of PKG and LZ+MYPT1 expression by hypoxia (Fig. 4a–d).To determine whether loss of LZ+MYPT1 expression in hypoxia-exposed cells is attributable to decreased levels of PKG expression or enhanced levels of ROS production, cells were transfected with PKG-siRNA and then exposed to hypoxia in the presence or absence of troloxor NAC. Under these conditions, trolox or NAC could not restore hypoxia-induced reduction in LZ+MYPT1 expression in PKG-siRNA transfected cells (Fig. 5a–g).This result suggests that ROS regulate LZ+MYPT1 expression only indirectly by modulating PKG expression in hypoxia.

Role of PKG and/or ROS in hypoxia-mediated decrease in MLCP activity

To further determine the mechanism for inactivation of MLCP in hypoxia, FPASMC transfected with PKG-siRNA or nonsilencingsiRNA were exposed to hypoxia in the presence and absence of trolox or NAC. Cell extracts were isolated, blotted onto membrane and probed with native MYPT1 or phospho-specific (Ser[695]) MYPT1 antibodies. The

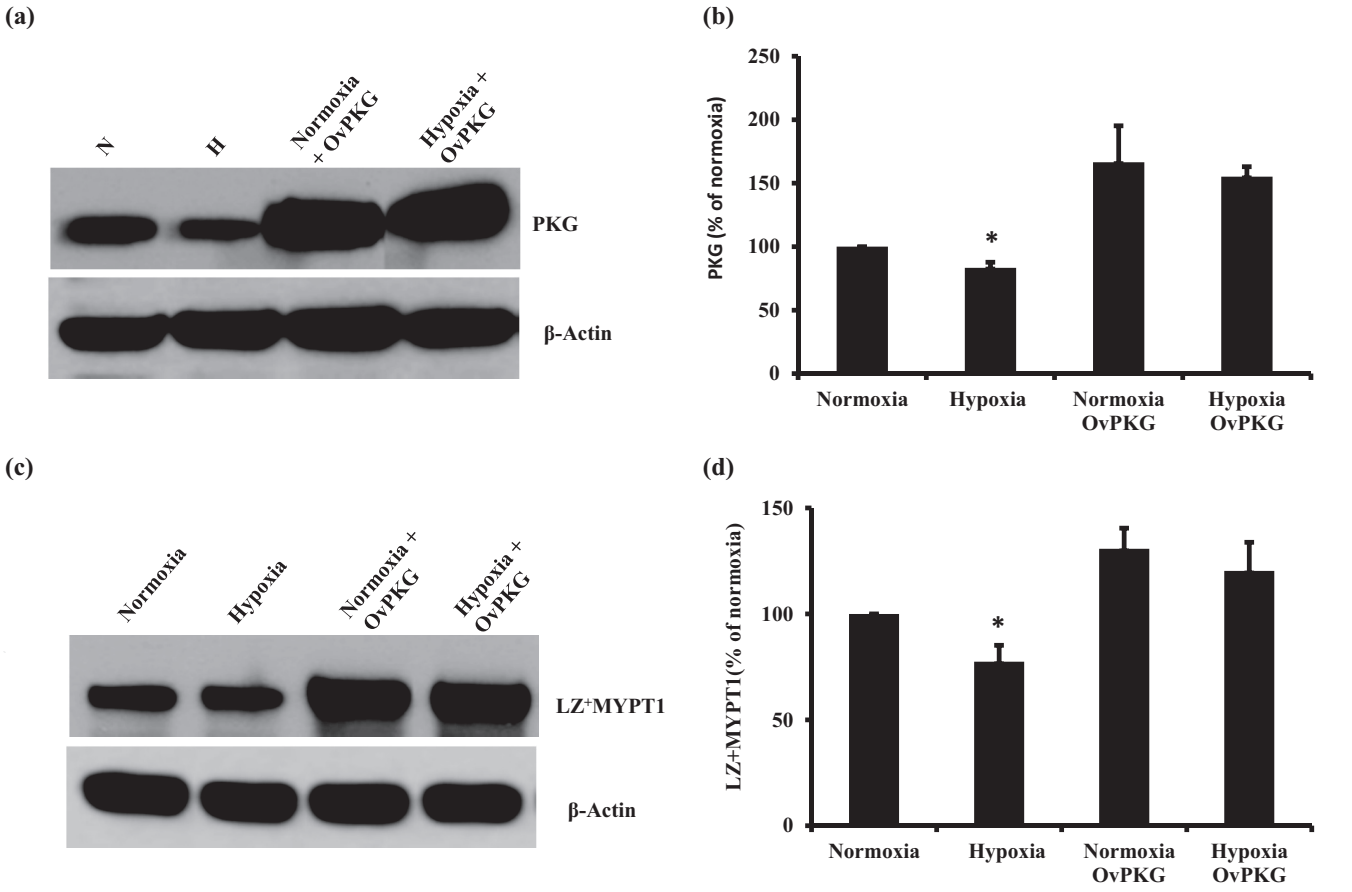

Figure 3: Overexpression of protein kinase G (PKG) inhibits LZ+MYPT1 down-regulation by hypoxia. Fetal pulmonary arterial smooth muscle cells FPASMC were transfected with a plasmid encoding a full-length PKG1α tagged with green fluorescent protein (GFP). PKG-–GFP GFP-expressing cells were exposed to acute hypoxia (4 h) and then expression levels of PKG (a) and LZ+MYPT1 (c) were analyzed. Panels b and d are the quantified levels of PKG and LZ+MYPT1 expression analyzed by immunoblot analysis. Data represent means + SE (n=3). *P<0.05 compared with respective control (normoxia).

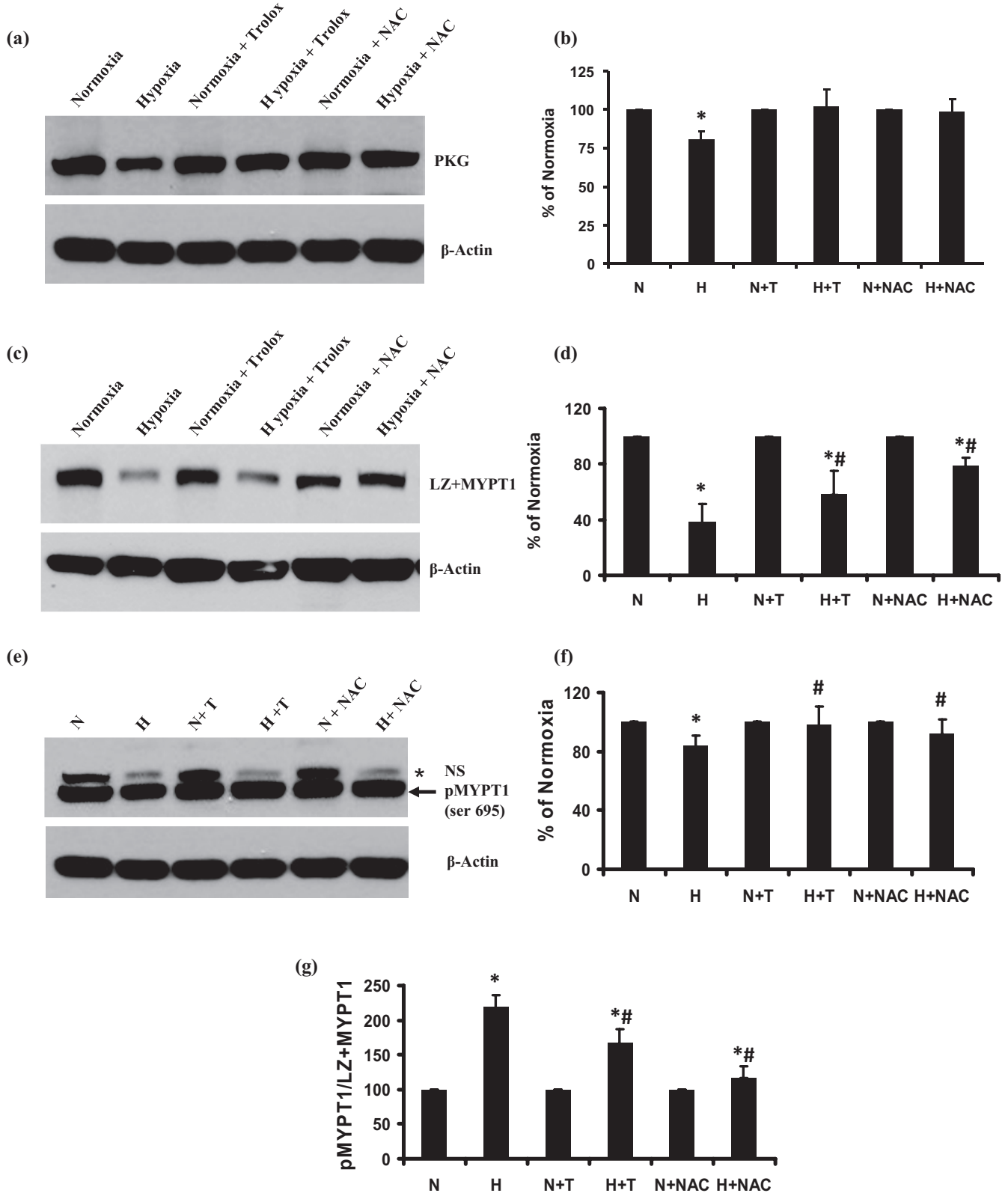

Figure 4: Hypoxia decreases LZ+MYPT1 expression via reactive oxygeb species-dependent protein kinase G (PKG) reduction. Cells were exposed to acute hypoxia (4 h) in the presence or absence of trolox (100 iM) or 1 mM N-acetyl cysteine. (a, c and e) Representative immunoblots showing the levels of PKG, LZ+MYPT1 and pMYPT1 (Ser^{695}), respectively. (b, d, f and g) Quantitative analysis of PKG (B) LZ+MYPT1 (D), pMYPT1 (Ser^{695}, F) and pMYPT1/LZ+MYPT1 (g). The values are represented as percent of control (normoxia) after normalizing the data to the respective â-actin. Data represent means+SE from at least three independent experiments. *P<0.05 compared with the respective control (normoxia) groups.

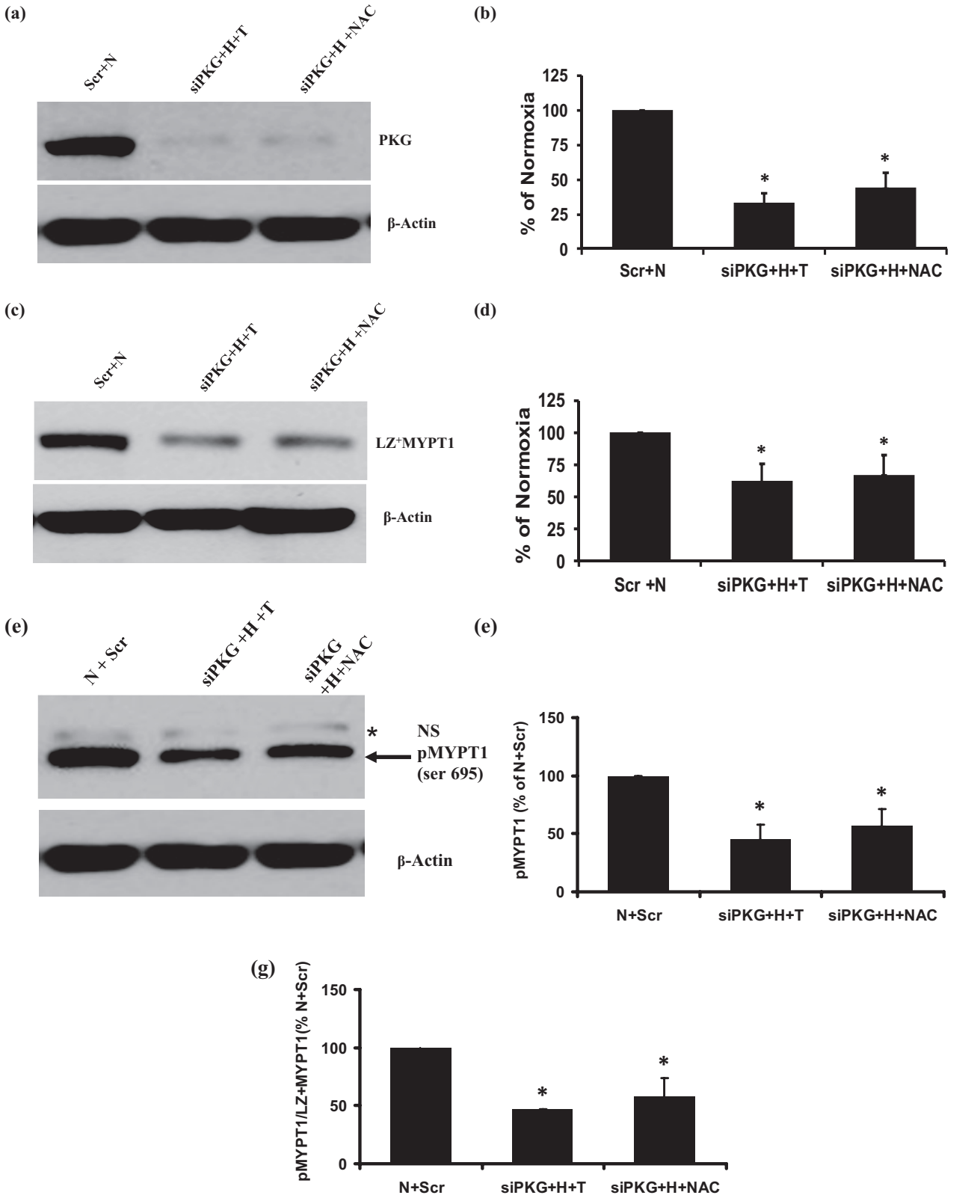

Figure 5: Hypoxia decreases LZ+MYPT1 expression via reactive oxygen species-dependent protein kinase G (PKG) reduction. Cells were transfected with siRNA specific for PKG or nonsilencings iRNA and then exposed to acute hypoxia (4 h) in the presence or absence of trolox (100 iM) or 1 mM NAC. (a, c and e) Representative immunoblots showing the levels of PKG, LZ+MYPT1 and pMYPT1 (Ser695), respectively. (b, d, f and g) Quantitative analysis of PKG (b) LZ+MYPT1 (D), pMYPT1 (Ser695, F) and pMYPT1/LZ+MYPT1 (g). The values represented as percent of control (normoxia) after normalizing the data to respective â-actin. Data represent means + SE from at least three independent experiments. *P<0.05 compared with the respective control (normoxia) groups.

phosphorylation of Ser^{695} represents the activated state of MYPT1. We found decreased (two-fold) levels of MYPT1 phosphorylation at Ser^{695} in both acute hypoxia (Fig. 4a–g) and PKG-siRNA transfected cells treated with trolox or NAC (Fig. 5a–g), but not in trolox- or NAC-treated cells not transfected with PKG-siRNA (Fig. 4e and f). This result suggests that hypoxia inactivates MLCP by decreasing PKG-mediated MYPT1 phosphorylation at Ser^{695}.

Overexpression of MYPT1 lacking LZ motif (LZ$^-$MYPT1) mimics the effects of hypoxia on PKG-mediated LZ$^+$MYPT and MLCP phosphorylation

FPASMC that were transfected with a MYPT1 ($^-$LZ) construct lacking the leucine zipper showed significantly decreased levels of endogenous LZ$^+$MYPT1 in normoxia, similar to the decreased levels following hypoxia (4h). Likewise, there was a significantly decreased (50% of control values) level of Ser^{695} phosphorylation in MYPT1 in ($^-$LZ) transfected cells as compared with control untransfected cells in normoxia. Because the MYPT1 ($^-$LZ) construct lacks the leucine zipper, PKG was unable to bind and phosphorylate it at Ser^{695} (Fig. 6a and c). There was a two-fold increase in the relative ratio of pMLC$_{20}$/MLC$_{20}$ in MYPT1 ($^-$LZ)-transfected cells as compared with untransfected cells (Fig. 6a and d), indicating decreased levels of MLCP activity. There was no significant change

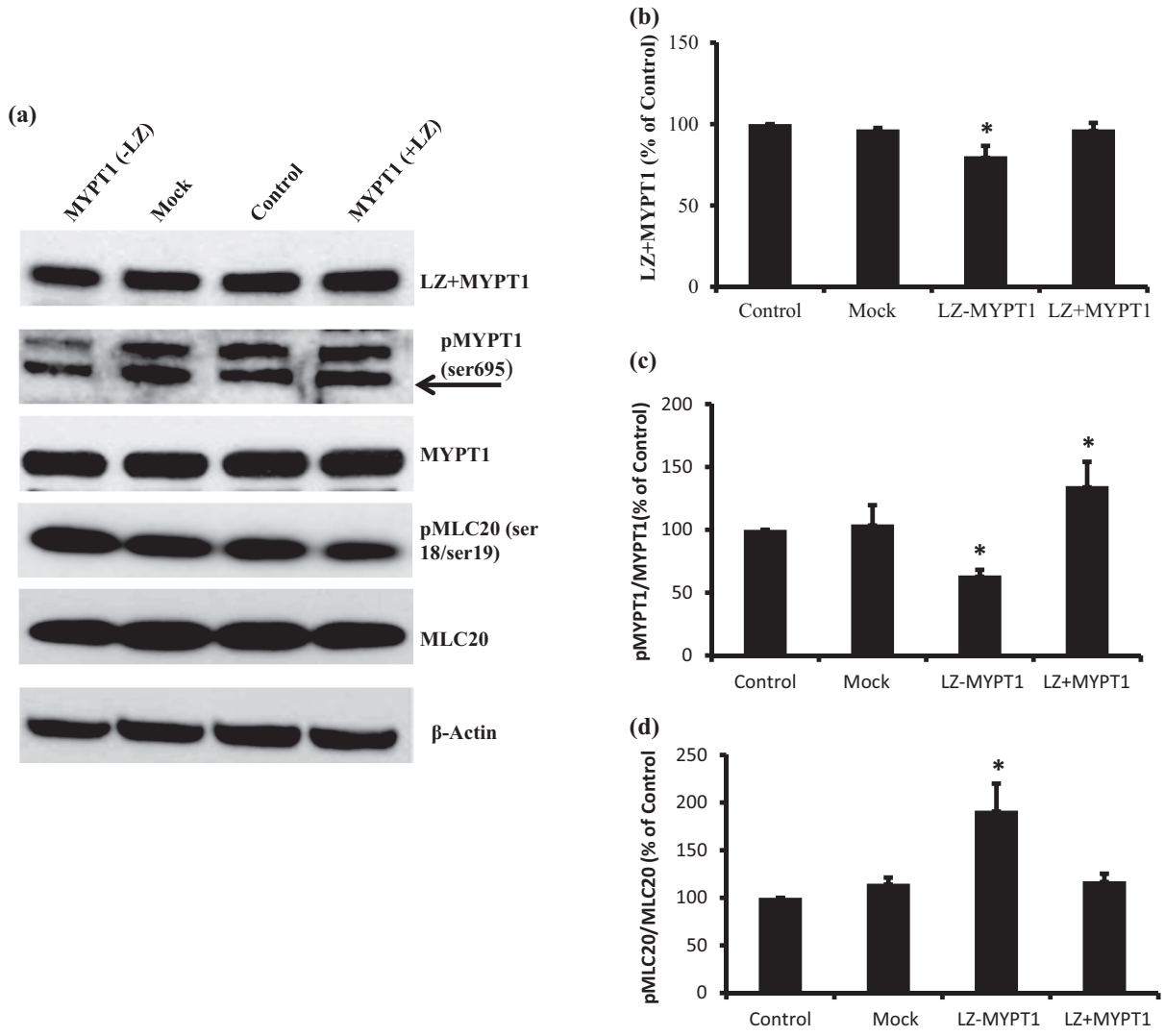

Figure 6: Deletion of the LZ motif in MYPT1 mimics hypoxia and decreases protein kinase G-mediated MYPT1 and MLC phosphatase phosphorylation. Fetal pulmonary arterial smooth muscle cells were transfected with MYPT1 expression vector without leucine zipper ($^-$LZ) or with Leucine zipper ($^+$LZ). Empty vector was used for mock transfections and untransfected cells as control. Cell lysates were immunoprobed with anti-LZ$^+$MYPT1, phospho (Ser^{695}) specific-MYPT1, native MYPT1, MLC$_{20}$, pMLC$_{20}$ and â-actin antibodies. (a) Representative Western blot probed with antibodies as indicated is shown. (b) Quantification of Western blot data for LZ$^+$MYPT1 relative to control (untransfected cells). (c) Quantitative analysis of pMYPT1 (Ser^{695})/MYPT1 levels. values are represented as percent of control ratios between pMYPT1 (Ser^{695}) and total MYPT1 after normalizing to their respective â-actin. (d) Normalized ratios of pMLC$_{20}$/MLC$_{20}$ as percent of control (untransfected cells) after normalizing the data to respective â-actin. Data represent means + SE from at least three independent experiments, *P<0.05 compared with the respective control (untransfected cells).

in the $pMLC_{20}/MLC_{20}$ratio (Fig. 6a and d) between mock-transfected cells and untransfected cells (Fig. 6a and d). These results indicate that the LZ domain of MYPT1 is important for PKG-mediated phosphorylation (Ser^{695}) of MYPT1, which subsequently activates MLCP, leading to dephosphorylation of $pMLC_{20}$, a step required for smooth muscle cell relaxation.

Hypoxia increases the interaction between RhoA or ROCK and LZ^+MYPT1, and overexpression of MYPT1 ($^-$LZ) mimics hypoxia

To determine the interaction between RhoA or ROCK and LZ^+MYPT1 in hypoxia, FPASMC were exposed to normoxia or hypoxia for 4 h, cell lysates prepared and immunoprecipitated with anti-PKG, anti-RhoA, anti-ROCK1 or anti-ROCK2 antibodies and then Western blotted with LZ^+MYPT1 antibodies. There was a two-fold increase in the interaction between LZ^+MYPT1 and RhoA or ROCK1/ROCK2 and decreased LZ^+MYPT1 interaction with PKG in FPASMC exposed to hypoxia (Fig. 7). In MYPT1 ($^-$LZ) overexpressing FPASMC, there was a significant decrease in interaction between PKG and LZ^+MYPT1 (Fig. 8a and b), whereas an increase (~two to four-fold) in interaction between LZ^+MYPT1 and RhoA, or ROCK1/ROCK2, was observed (Fig. 8c–f). In cells transfected with MYPT1 ($^+$LZ) expression vector, there was enhanced (~2.5-fold) interaction between PKG and LZ^+MYPT1 (Fig. 8a and b), accompanied by decreased levels of MYPT1 ($^+$LZ) interaction with RhoA or ROCK1/ROCK2 (Fig. 8c–f).

DISCUSSION

Our data show that acute hypoxia downregulates the expression levels of LZ^+MYPT1 by reducing PKG expression, decreases the interaction of LZ^+MYPT1 with PKG and promotes its interaction with ROCK1/ROCK2 in ovine FPASMC. These changes are accompanied by a decreased ratio of $pMLC_{20}/MLC_{20}$,indicative of increased smooth muscle contraction. Possible explanations for the change in binding affinity of PKG with LZ^+MYPT1 in acute hypoxia may be: (1) differential downregulation of PKG and LZ^+MYPT1, (2) increased affinity of RhoA, compared with PKG, to bind with LZ^+MYPT1; and/or (3) posttranscriptional modification of PKG such that its affinity to bind with the LZ domain of MYPT1 is decreased. The first two possibilities were examined by studying the effects of MYPT1 (LZ^+) or MYPT1 ($^-$LZ) overexpression on hypoxia-induced effects in SMC. Overexpression of LZ^+MYPT1 or its known upstream activator, PKG, reversed hypoxia-induced effects, whereas LZ^-MYPT1

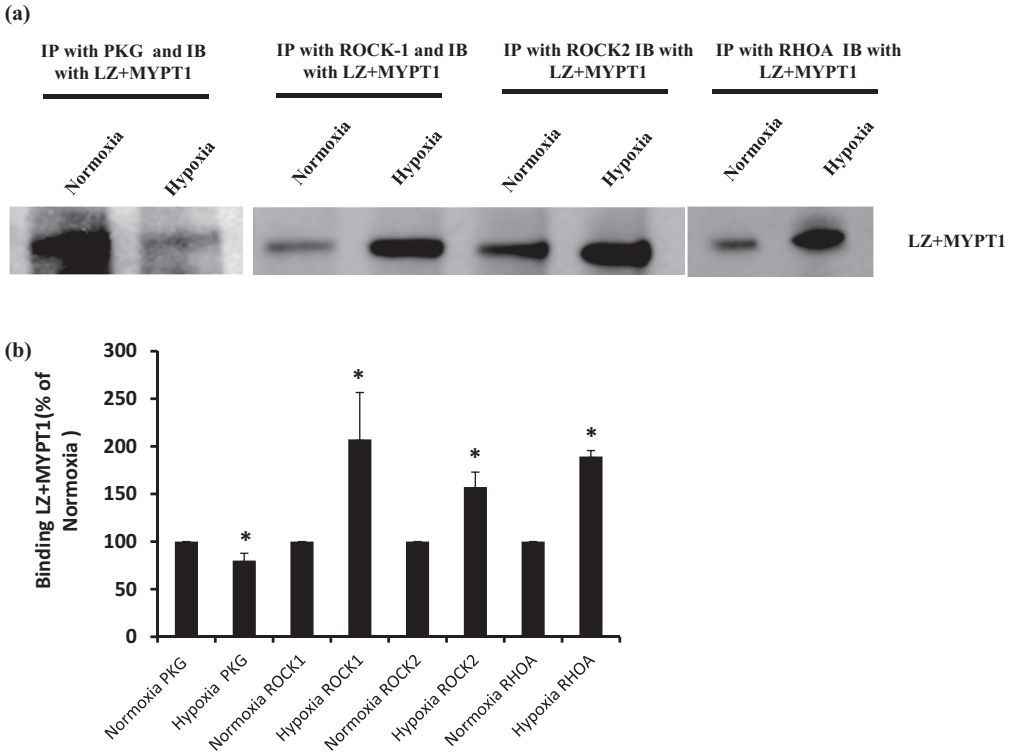

Figure 7: Hypoxia promotes interaction between RhoA or Rho kinase (ROCK) and LZ^+MYPT1. To determine the interactions between protein kinase G (PKG) and LZ^+MYPT1, fetal pulmonary arterial smooth muscle cells were exposed to normoxia or hypoxia for 4 h and cell lysates (200 ig) were immunoprecipitated and probed with the indicated antibodies. (a) Representative Western blot data showing reduced interaction between PKG and LZ^+MYPT1 and increased interaction between LZ^+MYPT1 with RhoA or ROCK1/ROCK2 following hypoxia exposure. (b) Quantitative analysis of Western blots from three independent experiments and values represented as percent of control (normoxia). Data represent means ± SE, *P<0.05 compared with control (normoxia).

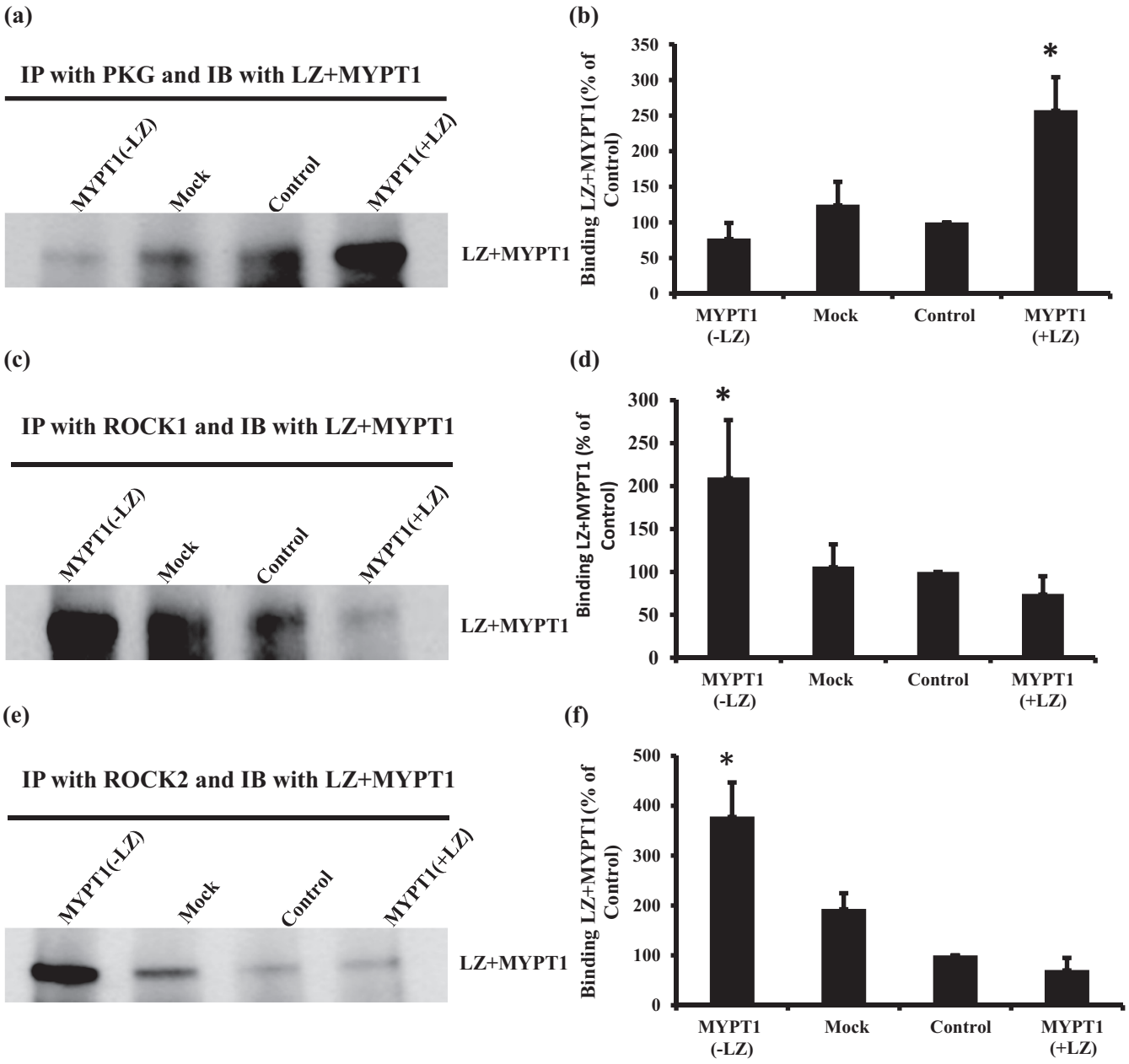

Figure 8: Overexpression of MYPT1 ($^{-}$LZ) mimics hypoxia-induced effects on protein kinase G (PKG) and LZ^{+}MYPT1 interactions. Cells were transfected with MYPT1 expression vector with ($^{+}$LZ) or without ($^{-}$LZ) leucine zipper. Empty vector was used as a control. Cell lysates (200 ìg) were immunoprecipitated with PKG, Rho kinase 1 (ROCK1) or Rho kinase 2 (ROCK2) antibodies and then immunoblotted with LZ^{+}MYPT1 antibody. Panel a: Western blot showing the interaction between PKG and LZ^{+}MYPT1. Cell lysates were immunoprecipitated with PKG antibody and then immunoblotted with LZ^{+}MYPT1 antibody. Panel b: Quantification of data in Panel a. Panel c: Western blot probed with LZ^{+}MYPT1 antibody showing interactions between LZ^{+}MYPT1 and ROCK1. Cell lysates were immunoprecipitated with ROCK1 antibody and then probed with LZ^{+}MYPT1 antibody. Panel d: Quantitative analysis of immunoblots in Panel c. Panel e: Representative immunoblot probed with LZ^{+}MYPT1 antibody to detect interaction between LZ^{+}MYPT1 and ROCK2 after immunoprecipitation with anti-ROCK2 antibodies. Panel f: Quantification of the data from panel e. Data represent means + SE from three to five independent experiments, *P<0.05 compared with the respective control (untransfected cells).

overexpression mimicked the effects of hypoxia. Based on these observations and those from our earlier studies,[13,15,16] we propose that hypoxia causes the downregulation of PKG expression, thereby leading to reduced LZ^{+}MYPT1 expression (see schematic, (Fig. 9)). This leads to suppression of PKG binding to LZ domain of MYTP1, decreased phosphorylation of MYPT1 at Ser^{695} and the inactivated state of MLCP. Lack of MLCP activation results in increased levels of $pMLC_{20}$ and contraction of SMC.

Previous studies have shown that ROS generated by hypoxia[17-20] may be responsible for both decreased PKG

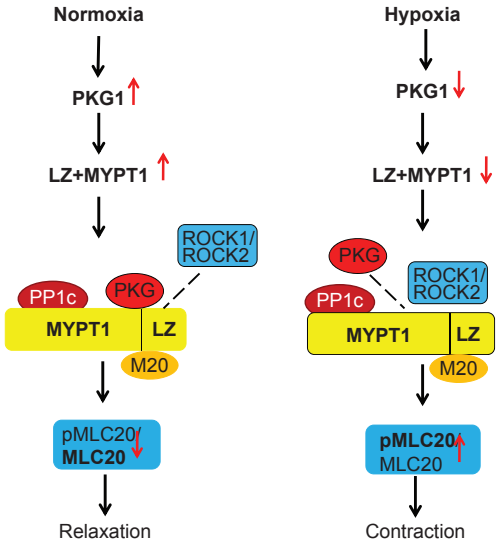

Figure 9: Schematic representation of signaling in smooth muscle cell in normoxia and hypoxia.

and LZ⁺MYPT1 expression. Our studies show that ROS modulates LZ⁺MYPT1 expression indirectly through modulation of PKG protein and activity levels. Although we found that hypoxia downregulates PKG expression in a ROS-dependent manner, PKG overexpression alone restored the levels of MYPT1 (⁺LZ) in cells exposed to hypoxia, and ROS scavengers failed to restore the expression levels of LZ⁺MYPT1 in PKG-deficient (knockdown) cells. These results suggest that hypoxia downregulates LZ⁺MYPT1 expression by reducing PKG levels through ROS, and not directly through ROS-mediated effects. Furthermore, our data also suggest that PKG-mediated activation of MLCP is required for the expression of LZ⁺MYPT1, as overexpression of MYPT (⁻LZ) mimics the effects of acute hypoxia on the expression of endogenous LZ⁺MYPT1.

SMC contractility is modulated by the ratio of phosphorylated MLC_{20} to unphosphorylated MLC_{20} ($pMLC_{20}/MLC_{20}$), and this ratio is regulated by the relative activities of MLCK/MLCP.[3,4] Activation and inactivation of MLCP depends on the ratio of site-specific phosphorylation of MYPT1 (Ser^{695}) to unphosphorylated MYPT1 (pMYPT1 Ser^{695}/MYPT1). In our study, hypoxia increased the $pMLC_{20}/MLC_{20}$ ratio, as expected. Increased pMYPT1/MYPT1 ratio and MYPT1 (⁺LZ) and PKG expression were associated with increased MLCP activity and smooth muscle relaxation in normoxia. Our data also showed that hypoxia decreases the interaction of PKG with MYPT1 and increases the interaction between LZ⁺MYPT1 and ROCK1/ROCK2. Surks et al. first reported that the binding of PKG to MYPT1 is mediated by the LZ⁺ motifs located at the N- and C-termini of the two proteins, respectively.[21] However, others have reported that the LZ⁺ motif of MYPT1 is not required for the binding of PKG to MYPT1.[22] Another group[23] used multiple biophysical techniques to fully characterize the interaction of the C-terminal region of MYPT1 with the N-terminal LZ⁺ motif of PKG. Their data showed that under physiological conditions, the LZ⁺ motif of PKG binds to the LZ⁺ motif of MYPT1 to form a heterodimer. When the LZ⁺ motif of MYPT1 is absent, the PKG LZ⁺ binds to the coiled-coil region and upstream segments of MYPT1 with the formation of a heterotetramer; however, there is no phosphorylation at Ser^{695} and no activation of MLCP.[22]

Regulation of expression levels of MYPT1 (⁺LZ) via the NO–cGMP–PKG pathway plays a key role in smooth muscle relaxation under a number of physiological and pathophysiological conditions.[14] For instance, in rats between postnatal days 6 and 12, expression of MYPT1 in portal veins switches from LZ (+) to LZ (-) isoforms,[11] which is concordant with a switch from cGMP-sensitive vascular relaxation to insensitivity to cGMP.[11,21,24,25] In the rat model of congestive heart failure, decreased relaxation of the aorta is associated with decreased expression of MYPT1 (⁺LZ).[7,24,26] Earlier studies have shown that continuous exposure of pulmonary vessels to nitric oxide leads to NO tolerance with decreased cGMP-induced PKG activity.[27,28] In these studies, it was reported that increased accumulation of cGMP led to downregulation of PKG protein expression and activity in a negative feedback manner. A similar study demonstrated that a reduction in MYPT1 (⁺LZ) expression, downstream of the NO–cGMP–PKG pathway, is involved in the development of NO tolerance and that this process is in part due to proteasome-dependent degradation of LZ⁺MYPT1.[27,29] Earlier studies have also shown that preservation of MYPT1 (⁺LZ) expression could prevent the decrease in cGMP-mediated vasodilatation in chronic heart failure.[24,26]

CONCLUSIONS

In summary, data presented in this study support our hypothesis that hypoxia stimulates multiple signaling pathways that act in a concerted manner to regulate SMC contraction. MYPT1 subunit of MLCP is the key regulatory protein in SMC contraction and relaxation, and dysfunctional MYPT1 signaling can contribute to the pathophysiology of a number of diseases;e.g., hypertension, gastrointestinal dysmotility, vasospasm and congestive heart failure.[7,30-32] Thus, we propose that strategies to restore MYPT1 (⁺LZ) expression, by selective inhibition of MYPT1 (⁺LZ) degradation and/or over expression of PKG or MYPT1 (⁺LZ), may provide a promising approach for the treatment of diseases linked to SMC dysfunction.

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