Hypoxia regulation of pulmonary vascular smooth muscle cGMP-dependant kinase by the ubiquitin conjugating system

Ramaswamy Ramchandran, Evgeny Pilipenko, Laura Bach, Aarti Raghavan, Sekhar. P. Reddy, J. Usha Raj

Online Supplemental Data:

MATERIALS AND REAGENTS:

Reagents: Quercetin, N-acetylcysteine, Trolox [(+)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid], IGEPAL CA-630, c-Myc-tag antibody, Protease inhibitor cocktail were obtained from Sigma(St. Louis, MO); MG132 was from Cayman Chemical (Ann Arbor, MI); 8-Bromo-cGMP Na salt, Actin, cGKIα from Santa Cruz Biotechnology Inc (Santa Cruz, CA), Ubiquitin (P4G7-H11, mouse monoclonal), PKG (rabbit polyclonal, KAP-PK002 and KAP-PK005) from Assay Designs (Ann Arbor, MI); PKG1 inhibitor, DT-3 from EMD Biosciences (San Diego, CA), Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA); 8-(2-Aminoethyl) thioguanosine-3´, 5´-cyclic monophosphate (8-AET-cGMP) agarose was purchased from BioLog (Breman, Germany).

ADDITIONAL METHODS:

Isolation of Fetal and Newborn Ovine pulmonary arteries: Fetal arteries (4-6th generation) were dissected and isolated from the lungs in a hypoxia chamber so as to maintain the hypoxic conditions of the fetal environment whereas arteries from newborn lamb lungs were isolated in room air.

Transfection of FPASMC: FPASMCs were transiently transfected with a plasmid encoding the myc-tagged full length PKG1α (12) or the empty vector using the Lipofectamine 2000 reagent (Invitrogen, CA). Briefly, 8 µg of the plasmid was mixed with 20 µl of lipofection reagent to form the liposomal complex in OptiMEM-I medium and applied on to plated cells in 5ml complete growth medium. The medium was replaced after 12-15 hours. Protein expression analysis and hypoxia treatment were performed after 36 h of transfection.

Immunoprecipitation: For immunoprecipitation, cell extracts containing same amount of protein were mixed with 2 μg of PKG1 (Assay Designs, rabbit polyclonal) or the PKG1α (Santa Cruz Biotech, goat polyclonal) antibody for 2 hours at 4 ºC. 40 μl of Protein A/G-PLUS-agarose beads was added and incubated for an additional 2 hour period. The immune complex was spun down at 3000 x *g* for 3 minutes and washed 3 times with RIPA buffer, boiled with SDS-PAGE sample buffer and resolved by SDS-PAGE (8% Tris-glycine acrylamide gel).

Affinity enrichment of PKG1: PKG1 proteins were enriched by binding to a cGMP analog 8-(2-Aminoethyl) thioguanosine-3´, 5´-cyclic monophosphate (8-AET-cGMP) agarose beads (19). Briefly, FPASMCs were exposed to normoxia or hypoxia for 3 hours and cell lysates prepared in cold buffer A [50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 % IGEPAL® CA-630 with protease inhibitor cocktail]. The cell extracts containing same amount of protein were mixed with 40 μl of 8-AET-cGMP agarose beads (ligand density ~6.5 μmol/ml BioLog, Germany) and incubated for 1 hour at 4 ºC with mild agitation. The beads were spun down at 2000 x *g* for 5 minutes in a centrifuge. The beads were washed thrice and the 'bound' proteins eluted with buffer A containing 25 mM cGMP. The bound and unbound protein fractions were immunoprecipitated with PKG1α antibody and subjected to Western analysis. The blot was probed with ubiquitin-specific monoclonal antibody and subsequently reprobed with PKG1α antibody. Loading controls were verified by probing cell lysates with actin and PKG1 antibodies.

Statistics: Data are expressed as mean ± SEM. Significant differences between groups was determined using the Student's *t* test. Values of $P < 0.05$ were considered significant.