Protein kinase C-α and Arginase I mediate Pneumolysin-induced pulmonary endothelial hyperpermeability.

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Supplemental Data

Supplemental Materials and Methods.

Biochemicals. Ro32-4032 and BEC (*S*-(2-boronoethyl)-L-cysteine) were from Calbiochem (San Diego, CA). Cyclic hTNF-derived TIP peptide[23] was from Bachem (Bubendorf, Switzerland). Mouse anti-human MLC mAb was from Sigma-Aldrich (St Louis, MO), rabbit anti-human di-p(Thr18/Ser19)-MLC, mouse anti-human GAPDH and beta actin mAb were from Cell Signaling Technology (Beverly, MA). Rabbit anti-human arginase I and arginase II polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-total human PKC-α and anti-p(Thr638) PKC-α mAb were from BD Transduction Labs (Lexington, KY). Anti-mouse and anti-rabbit secondary antibodies conjugated to horse radish peroxidase were from Sigma-Aldrich. Enhanced chemiluminescence (ECL) detection kit was from Pierce (Rockford, IL).

Immunofluorescence microscopy. As primary antibodies for microtubule staining, we used monoclonal mouse antibodies against β-tubulin (ICN, USA) (1:200) and monoclonal mouse antibodies against acetylated tubulin (Accurate Chemicals, USA) (1:100). Anti-mouse antibodies conjugated to the fluorescent dyes Alexa-488 (Molecular Probes, USA) (1:100) were used as secondary antibodies. Prior to immunofluorescence staining, HPAEC grown on glass coverslips were fixed for 10 min with a 1.5% solution of glutaraldehyde (Sigma, USA) in phosphate-buffered saline (PBS), pH 6.8 (Sigma, USA) and washed three times with PBS (each washing session lasted 10 min). Fixed cells were permeabilized with 0.1% Triton X-100 (Sigma, USA) in PBS for 15 min and washed three times with PBS for 10 min. To avoid background fluorescence, prior to staining with antibodies cells were treated with a 0.2% solution of sodium borohydride (NaBH4) (Sigma, USA) in PBS (10 min, three times) and washed 3 times with PBS for 10 min. The next step included incubation of cells with primary (30 min, 37°C) and secondary (30 min, 37°C) antibodies. The coverslips were mounted on slides in water/glycerol mixture (1:1) as a priming medium. Prior to the assays, cover glass edges were sealed with nail polish for better fixation of the samples. Immunofluorescence stainings of endothelial monolayers were examined under a Nikon Eclipse TE2000 microscope (Nikon Intech Co., Japan), supplied with a 60/1.4 objective. The most spread cells were selected in order to ensure better visualization of the cell structures. Images were recorded using Hamamatsu ORCA-2 (Hamamatsu Photonics, Japan) digital cooled CCD camera supported with MetaView software (Universal Imaging, USA). The resolution of 12-bit digital images was 9 pixel/μm. Image processing was performed using ImageJ software and Adobe Photoshop 7.0 (Adobe Inc., USA) software**.**

Rac1 and RhoA Activation Assays. RhoA and Rac1 activities of HL-MVEC were determined using absorbance-based G-LISA Rac1 and RhoA activation assay kits, according to the manufacturer's instructions (Cytoskeleton, Inc., Denver, CO).

Measurement of PKC-α activation in HL-MVEC. PKC-α activation in HL-MVEC was assessed as described previously (E1).

Measurement of transendothelial electrical resistance (TER). TER in HL-MVEC monolayers (ECIS system 1600R, Applied Biophysics, Troy, NY) was measured as described previously (E1).

Ca^{2+} influx measurements. HL-MVEC were transduced with adenovirus encoding the Ca^{2+}-sensitive photoprotein aequorin, activated 24h later by incubating the cells in Ca^{2+}-

free DMEM (Biosource) containing coelenterazine (Sigma). Subsequently, loading media was replaced with phenol-free DMEM without coelenterazine. Cells were placed in a luminescence plate reader (Lumistar Galaxy) and challenged with PLY (20-40 ng/ml). Luminescence was recorded over time as previously demonstrated (E2).

Arginase activity and expression. Arginase activity in HL-MVEC cell lysates was assayed by measuring urea produced from L-arginine, as described previously (E3).

Protein extracts were obtained from either HL-MVEC lysates or from frozen lungs pulverized and homogenized in RIPA lysis buffer (Upstate), containing protease inhibitors. Soluble protein extracts from cell lysates or tissue homogenates were subjected to SDS-PAGE electrophoresis, transferred to polyvinylidene fluoride (PVDF) membranes and reacted with anti-arginase I or II (1:250, Santa Cruz Biotechnology, Inc.) primary antibodies, followed by donkey anti-rabbit horseradish peroxidase-labeled secondary antibody (1:6000, GE Healthcare). Membranes were then stripped and reprobed with β-actin antibody to assess level of protein loading. Protein expression was determined using densitometry analysis of films.

Supplemental Reference list.

E1. Xiong C, Yang G, Kumar S, Aggarwal S, Leustik M, Snead C, Hamacher J, Fischer B, Umapathy NS, Hossain H, Wendel A, Catravas JD, Verin AD, Fulton D, Black SM, Chakraborty T, Lucas R. The lectin-like domain of TNF protects from Listeriolysin-induced hyperpermeability in human pulmonary microvascular endothelial cells - a crucial role for Protein Kinase C-alpha inhibition. *Vascul Pharmacol.* 2010;52(5-6):207-213.

E2. Church JE, Fulton D. Differences in eNOS activity because of subcellular localization are dictated by phosphorylation state rather than the local calcium environment*. J Biol Chem*. 2006;281:1477-1488.

E3. Romero M, Platt D, Tawfik H, Labazi M, ElRemessy AB, Bartoli M, Caldwell RB, Caldwell RW. Diabetes-induced coronary vascular dysfunction involves increased arginase activity. *Circ. Research*, 2008, 102;95-102.