Materials and Methods

Cyclophilin A is required for Angiotensin II-induced p47phox Translocation to Caveolae in Vascular Smooth Muscle Cells: Role in Reactive Oxygen Species Production

Nwe Nwe Soe, Mark Sowden, Padmamalini Baskaran, Elaine M. Smolock, Yeonghwan Kim, Patrizia Nigro, Bradford C. Berk

Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY, 14642, USA. (N.N.S., M.S., E.M.S., Y.K., B.C.B.)
School of Pharmacy, College of Health Sciences, University of Wyoming, 1000 East University Avenue, Laramie, WY 82071 (P.B.)
Laboratorio di Biologia Vascolare e Medicine Rigenerative Centro Cardiologico Monzino-IRCCS, via Parea 4, 20138 Milano, Italia (P.N.)

Running title: CyPA regulates p47phox translocation to caveolae

Corresponding author:
Bradford C. Berk, MD, PhD
Aab Cardiovascular Research Institute, University of Rochester
Box CVRI, 601 Elmwood Avenue, Rochester, NY 14642
Phone: (585)275-3407, Fax: (585)273-1059
E-mail: bradford_berk@urmc.rochester.edu

Reagents
Reagents used were: Human AngII (Calbiochem), CyclosporineA (Sigma Aldrich), VAS2870 (Enzo Life Sciences), Diphenylene iodonium (Sigma Aldrich), methyl-β-cyclodextrin (Sigma Aldrich), cholesterol-water soluble (Sigma Aldrich), Rottlerin (Calbiochem), Cytochalasin B (Sigma Aldrich), Protein G or A agarose beads (Roche), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes).
Antibodies used were: rabbit anti-p47phox (Millipore & Santa Cruz Biotechnology), mouse anti-CyPA (Santa Cruz Biotechnology), rabbit anti-CyPA (Enzo Life Sciences), mouse Smooth muscle cell actin (clone A4, Santa Cruz Biotechnology), mouse anti-Actin (Santa Cruz Biotechnology), rabbit anti-caveolin 1 (Cell Signaling Technology) and mouse anti-FlagM2 (Sigma), mouse anti-HA (Covance) and mouse anti-GAPDH (Millipore).

Isolation and culture of Aortic Smooth Muscle Cells
Aortic smooth muscle cells from rats (RASMC) or mice (MASMC) from WT, *Ppia*-/- or overexpressed Flag-CyPA were isolated by enzymatic digestion and maintained in Dulbecco`s modified Eagle`s medium (DMEM) containing 10% fetal bovine serum (FBS) as described previously[1]. Passages 4 to 6 of MASMC or 6 to 12 RASMC at 70% to 80% confluence were growth arrested by incubation in 0.3% FBS containing DMEM for 24 hour followed the indicated experiments. HeLa and HEK293 cell lines were maintained in 10% FBS, 100µg/ml streptomycin and 100IU/ml penicillin. All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Angiotensin Type 1 Receptor (AT1R) stably expressed HeLa cell line generation
A stable HeLa cell line expressing HA-epitope tagged Angiotensin II receptor type I (HA-AT1R) was generated by transfection (Fugene6, Promega) of a C-terminally HA epitope tagged AT1R cDNA in pcDNAIII. The line was selected under G418 (1mg/ml) and maintained in DMEM containing 10%FBS.

Construction of recombinant lentivirus and VSMC transduction
pLV-CMV-IRES-GFP is an HIV-1 based lentiviral expression vector that allows simultaneous expression of CyPA or p47phox cDNA (and mutants thereof) from the CMV promoter and EGFP by means of an IRES element. Infectious viral particles were generated by co-transfection of the transgene, with plasmids expressing viral gag/pol genes (psPAX2) and VSV-G coat protein (pMD2.G) into HEK293T cells using Fugene6 (Promega). 48 hour post-transfection viral containing supernatant were collected, filtered through 0.45µm cellulose acetate filters and stored in aliquots at -80°C. For transduction, VSMC were plated at subconfluence in 6 well culture plates and spin-oculated (1500 x g for 1.5 hours at room temperature) with virus in the presence of polybrene (8 µg/ml; Sigma Aldrich). Cells were incubated for 48 hours and changed the media to serum free DMEM 1 hour before AngII stimulation.

Plasmids

Flag-tagged WT-CyPA and R55A-CyPA plasmids were expressed from pIRES2-EGFP (Clontech). HA-tagged p47phox (Open Biosystems 3983481) or PX-deletion mutants were generated using standard techniques. All cDNA sequences were confirmed at the University of Rochester Genomics Research Center.

Cell Culture and Transfection

Approximately 80% confluent AT1R-Hela cells grown on 60mm dish were transfected with 6μg of cDNA/dish using Fugene 6 (Promega). 48 hours after transfection, cells were serum starved with serum free DMEM medium for 2 hours and then stimulated with AngII in the indicated experiments.

Measurement of reactive oxygen species by flow cytometry

VSMC were collected by trypsinization and incubated with 2 mmol/L 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen) in 3% FBS/PBS at 37°C for 30 minutes. Cells were centrifuged, washed and incubated again with AngII at 37°C according to the experimental design. Cells were centrifuged, washed and resuspended in 3% FBS/PBS followed by flow cytometry (Accuri® C6). The data were analyzed using FlowJo software (TreeStar Inc.).

Subcellular fractionation

VSMC were lysed in cell fractionation buffer (250 mmol/L Sucrose, 20 mmol/L HEPES, 10mM KCL, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1:1000 protease cocktail Inhibitor (Sigma) and lysates passed 20 times through a 25G needle and then centrifuged at 3000g for 10 minutes. The pellet was collected as the nuclear fraction and resuspended in nuclear lysis buffer (cell fractionation buffer with 10% glycerol, 0.1% SDS). The supernatant was transferred and centrifuged at 8000g for 15 minutes to separate the mitochondria (pellet) and the cytosolic fraction. The final cytosolic fraction was centrifuged at 100000 g for 1 hour. The membrane fraction (pellet) and the cytosolic fraction (supernatant) were collected. CyPA and p47phox localization in different cell fractions was assessed by SDS-PAGE.

Sucrose Density Gradient Centrifugation

Sucrose density gradient fractionation was performed as previously described[2] with minor modifications . Briefly, VSMC were washed with PBS and scraped into 10 ml of PBS, pelleted, and resuspended in 1 ml buffer 1 (500 mmol/L Na_2CO_3 pH11, 25 mmol/L MES pH 6.5, 150 mmol/L NaCl, and 1:1000 protease inhibitor mixture solution (Sigma), homogenized (30 strokes in a Dounce Homogenizer) and (3 x 10-s bursts, Ultrasonic homogenizer). The homogenate was placed in a 17ml-ultracentrifuge tube and was adjusted to 45% sucrose by adding 1.5 ml of lysis buffer and 2.5 ml of buffer 2 (90% sucrose, 25 mmol/L MES, 150 mmol/L NaCl, and 1:1000 protease inhibitor mixture solution). 4ml of buffer 3 (35% sucrose, 250 mmol/L Na_2CO_3 pH11, 25 mmol/L MES, 150 mmol/L NaCl, and 1:1000 protease inhibitor mixture solution) and 3 ml of buffer 4 (5% sucrose, 250 mmol/L Na_2CO_3 pH11, 25 mmol/L MES, 150 mmol/L NaCl, and 1:1000

protease inhibitor mixture solution) were layered on top of it and the samples were centrifuged at 28,000 rpm for 22 hours in a SORVALL Discovery 100S ultracentrifuge equipped with Spin-630 (17ml) rotor.12 x 1ml fraction were collected from the top to the bottom of each tube. The proteins were precipitated by adding 1ml of 20% trichloroacetic acid for each fraction and incubated on ice for 30 minutes, then centrifuged at 8000 rpm at 4°C for 15 minutes. The supernatants were carefully discarded and the pellets were washed with 500µl cold acetone and centrifuged at 8000 rpm for 5 minutes. The pellets were dried and resuspended with 50µl of 2.5X SDS-PADE loading buffer. An equal volume of each fraction was subjected to the Western blot.

Actin fractionation

F-actin and G-actin were separated from VSMC as described previously[3]. The cells were washed with PBS and then scraped into 10 ml PBS and centrifuged 1000 rpm for 10 minutes. The pellets were resuspended with lysis buffer A (20 mmol/L Tris/HCL, pH 7.5, 1% Triton X-100, 5 mmol/L EGTA, 1 mmol/L PMSF, 1:1000, protease inhibitor and incubated on ice for 20 minutes and then centrifuged 10 minutes, 140000 rpm. The supernatants were collected (cytosolic fraction, G-actin). The pellets were lysed again with Buffer B (10 mmol/L Tris/HCL, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 1 mmol/L sodium deoxycholate, 2 mmol/L EGTA, 1 mmol/L PMSF, 1:1000, protease inhibitor), incubated on ice for 30 minutes and centrifuged for 30 minutes at 140000 rpm. The supernatant was cell cytoskeleton (F-actin). Equal amount of each fraction was subjected to the Western blot.

Immunofluorescence

VSMC plated on 35 mm dishes were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. The fixed cells were blocked with 3% bovine serum albumin (BSA) plus 5% goat serum for 1 hour and then incubated with primary antibody (rabbit anti-p47phox, 1:50 & mouse anti-CyPA, 1:200) overnight at 4^{o}C. After washing, cells were incubated with secondary antibodies (Alexa-fluor 546 or 488; Invitrogen) and /or Fluorescein Isothiocyanate Labeled (FITC) conjugated phalloidin (Sigma). Images were captured using a BX51 Epi-Fluorescence Microscope (Olympus).

Immunoprecipitation and Western Blotting

VSMC were lysed in buffer (1% NP-40, 25 mmol/L Tris, pH 7.5, 50 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 137 mmol/L NaCl, 10% glycerol, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, and 10 µg/mL leupeptin), scraped off the dish, and centrifuged at 14 000 rpm at 4°C for 10 minutes, and protein concentrations were determined by Bradford protein assay. Lysates containing equal amounts of soluble proteins were incubated with antibody overnight at 4°C. Antibody complexes were collected by incubation with protein G or A agarose for 2 hours. Precipitates were washed 3 times in lysis buffer and then resuspended in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed. After incubation in blocking solution (5% BSA), membranes were incubated with primary antibodies for overnight at 4°C or 2 hours at room temperature. Excess primary antibody was removed by washing the membranes in PBS containing 0.01% Tween 20. The blots were incubated with appropriate secondary antibodies for 1 hour. The membranes were

washed, and proteins were detected by the ECL system (Amersham Life Science or Immobilon™ Western, Millipore).

Statistical Analysis

Experiments were repeated at least 3 times. Results are presented as mean±SEM and compared by Student's t test. $p<0.05$ was considered significant.

References

1. **Satoh K, Matoba T, Suzuki J, O'Dell MR, Nigro P, Cui Z, Mohan A, Pan S, Li L, Jin ZG, Yan C, Abe J, Berk BC. Cyclophilin a mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation. *Circulation*. 2008;117:3088-3098**
2. **Lungu AO, Jin ZG, Yamawaki H, Tanimoto T, Wong C, Berk BC. Cyclosporin a inhibits flow-mediated activation of endothelial nitric-oxide synthase by altering cholesterol content in caveolae. *J Biol Chem*. 2004;279:48794-48800**
3. **Lv P, Miao SB, Shu YN, Dong LH, Liu G, Xie XL, Gao M, Wang YC, Yin YJ, Wang XJ, Han M. Phosphorylation of smooth muscle 22alpha facilitates angiotensin ii-induced ros production via activation of the pkcdelta-p47phox axis through release of pkcdelta and actin dynamics and is associated with hypertrophy and hyperplasia of vascular smooth muscle cells in vitro and in vivo. *Circ Res*. 2012;111:697-707**