Circ J 2018; 82: 1195–1204
doi:10.1253/circj.CJ-17-0732

Neuroprotection Mediated by Upregulation of Endothelial Nitric Oxide Synthase in Rho-Associated, Coiled-Coil-Containing Kinase 2 Deficient Mice

Yukio Hiroi, MD, PhD; Kensuke Noma, MD, PhD; Hyung-Hwan Kim, PhD; Nikola Sladojevic, MD, PhD; Corey E. Tabit, MD; Yuxin Li, MD, PhD; Guray Soydan, MD, PhD; Salvatore Salomone, MD, PhD; Michael A. Moskowitz, MD; James K. Liao, MD

Supplementary File 1

Supplementary Methods

Fasudil was a gift from Asahi-Kasei Pharmaceuticals, Inc. (Tokyo, Japan). Plasmid containing wild-type (WT) eEF1A1 cDNA were described previously.[S1] Site-specific mutation was introduced using the QuikChange-XL kit (Stratagene), following confirmation by sequencing.

Animals

All mice used are congenic strains on a C57BL/6 background. Animal protocols were in compliance with NIH Guidelines for the Care and Use of Laboratory Animals. All protocols pertaining to experimentation with animals in this study were approved by the Standing Committee on Animals at Harvard Medical School and the Institutional Animal Care and Use Committee at the University of Chicago. Age-matched male littermates (10–20 weeks) were used for experiments. Both Rho-associated kinase (ROCK)$1^{+/-}$ and $ROCK2^{+/-}$ mice were generated, as described.[S2,S3] Endothelial nitric oxide synthase $(eNOS)^{-/-}$ $ROCK1^{+/-}$ and $eNOS^{-/-}ROCK2^{+/-}$ mice were generated by mating $eNOS^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME, USA) with $ROCK1^{+/-}$ or $ROCK2^{+/-}$ mice. Tie2-Cre transgenic mice, (Jackson Laboratory) were mated with $ROCK2^{flox/flox}$ to generate endothelial-specific ROCK2 knockout mice (EC-$ROCK2^{-/-}$).

Ischemic Stroke Model

WT, Rho-associated kinase (ROCK)$1^{+/-}$, $ROCK2^{+/-}$, $eNOS^{-/-}$, $eNOS^{-/-}ROCK1^{+/-}$, $eNOS^{-/-}ROCK2^{+/-}$, control (Tie2-Cre) and EC-$ROCK2^{-/-}$ mice were subjected to transient intraluminal occlusion of the middle cerebral artery (MCA) as described.[S4,S5] Briefly, mice were anesthetized with 2% halothane in 70% N_2O and 30% O_2, and then maintained on 1% halothane in a similar gaseous mixture. Transient focal cerebral ischemia was performed using an 8–0 nylon monofilament coated with silicone. This was introduced into the internal carotid artery via the external carotid artery and then advanced 10 mm distal to the carotid bifurcation to occlude the MCA. Laser Doppler flowmetry of cerebral blood flow (CBF) was used to verify that occlusion was successful (<20% baseline value). MCA occlusion lasted for 1 h, after which the filament was withdrawn and the brain was reperfused for 22 h.

Cerebral infarct volume and decreased neurological deficit score (NDS) were measured after reperfusion. Infarction area was measured in 2-mm-thick coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride (TTC), and quantitated using an image-analysis system (Bioquant IV; R&M Biometrics, Nashville, TN, USA). Cerebral infarct volume was determined by summing the infarcted areas. NDS was determined, as described previously.[S6] Briefly, a grading scale of 0–4 was used; 0=no observable neurological deficit; 1=failure to extend right forepaw; 2=circling to the contralateral side; 3=loss of walking or righting reflex; and 4=comatose or moribund.

Measurement of Absolute CBF

Absolute CBF was quantified using a previously described indicator fractionation technique.[S4] Briefly, the right jugular vein and left femoral artery of anesthetized mice were cannulated. The mice then received 1 mCi of N-isopropyl-[methyl 1,3-^{14}C]-p-iodoamphetamine in the right jugular vein, as a bolus.[S7] In total, 100 μL of arterial blood was collected from the left femoral artery for 20 s (0.3 mL/min). The animal was then decapitated and the whole brain was removed and immediately frozen in isopentane solution chilled on dry ice. The frozen brains were weighed and digested with Scintigest (Fisher Scientific, Hampton, NH, USA) at 50°C for 6 h. Scintillation fluid and H_2O_2 were added to the sample, and the samples were shaken for 12 h. The N-isopropyl-[methyl 1,3-^{14}C] p-iodoamphetamine in the blood and brain samples were measured by liquid scintillation spectrometry (RackBeta 1209; Pharmacia-Wallac, Oy, Finland). CBF was calculated as described: CBF (mL/100 g per min)=[brain count (cpm)×0.3 (mL/min)/blood count (cpm)×brain weight (g)]×100.[S7,S8]

Cell Culture For isolation of mouse brain microvascular endothelial cells (MBECs), 12- to 14-week-old mice were used. Briefly, brains were isolated and gray matter was carefully dissected, minced in Hanks balanced salt solution (HBSS; Invitrogen Corp, CA, USA), and gently homogenized in a Dounce type homogenizer. The microvessels were separated from erythrocytes with Percoll gradient (GE Healthcare Bio-Sciences, PA, USA). Microvessels were then digested in 1 mg/mL collagenase/dispase (Roche, IN, USA) for 30 min at 37°C in HBSS. Primary BMECs were grown in plates coated with collagen IV (BD Bioscience, NJ, USA) in full endothelial cells (ECs) medium purchased from ScienCell Research Laboratories (Carlsbad, CA,

USA) at 37°C in a humid atmosphere with 5% CO_2. To obtain 99% pure culture, we purified BMECs with puromycin on day 1 for 2 days. Bovine aortic ECs (BAEC) were cultured in DMEM, 10% FBS, and antibiotics.

Mouse heart ECs (MHEC) were isolated using Dynabeads[S9] and cultured in DMEM (Invitrogen), 20% FBS, and antibiotics. Primary human brain microvascular ECs (HBMEC) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and were used at passage 1 or 2. They were cultured in EC medium as recommended by the manufactured at 37°C in a humid atmosphere with 5% CO_2.

Western Blotting

ECs lysates were prepared using Lysis Buffer (Cell Signaling) containing protease inhibitors (EMD Biosciences). These samples were centrifuged and the supernatant was collected. Protein concentration was measured by using the Bradford method (Bio-Rad). In total, 20 μg of protein from each sample was separated on SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed using antibodies to ROCK1 (1:250 dilution; BD Biosciences), ROCK2 (1:1,000 dilution; BD Biosciences), eNOS (1:1,000 dilution; BD Biosciences), phospho-specific eNOS (1:1,000 dilution; Cell Signaling), MBS (1:5,000 dilution; Covance Inc.), phospho-specific Thr^{853} MBS (1:1,000 dilution), eEF1A1 (1:500 dilution; Santa Cruz Biotechnology), phospho-specific Thr^{432} eEF1A1 (1:250 dilution), β-actin (1:5,000 dilution; Sigma-Aldrich) and GAPDH (1:1,000 dilution; Cell Signaling). Immunodetection was performed using an anti-mouse secondary antibody (1:5,000 dilution; GE Healthcare Bio-Sciences) or anti-rabbit secondary antibody (1:5,000 dilution; GE Healthcare Bio-Sciences) and the Enhanced Chemiluminescence kit (Thermo Scientific). Images were obtained with a ChemiDoc™ MP System (Life Science Research, CA, USA).

Assessment of Endothelial-Dependent Vascular Reactivity

To determine whether a deficiency for ROCK1 or ROCK2 is associated with improved endothelial function, we measured vascular reactivity in male WT, $ROCK1^{+/-}$, $ROCK2^{+/-}$, control, and EC-$ROCK2^{-/-}$ mice. The mice were sacrificed and aortic rings were quickly mounted on an isometric myograph (610M; Danish Myo Technology, Aarhus, Denmark) in physiological solution (composition, mmol/L: NaCl, 118; KCl, 4.6; $NaHCO_3$, 25; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 1.25; glucose, 10; EDTA, 0.025; pH 7.4 at 37°C). After equilibration, contractile responses (i.e., wall tension) were recorded. Aortic rings were exposed to a 100 μmol/L KCl-depolarizing solution and, after washout, they were then exposed to a range of concentrations of phenylephrine (10^{-10} to 10^{-6} mol/L). Caution was taken to avoid damaging the endothelium, and the functional integrity of this structure was confirmed by the dose responses to ACh (10^{-9} to 10^{-6} mol/L; Sigma) and to the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (10^{-5} to 10^{-3} mol/L, Sigma). The maximal contraction evoked by phenylephrine was considered the baseline when subsequent vasorelaxations were evoked. Vasorelaxation is expressed as a percentage reduction in contraction. Endothelium-independent vasorelaxation was determined by response to sodium nitroprusside (SNP; 10^{-10} to 10^{-6} mol/L).[S10]

DAF-FM Diacetate Staining

Fresh brains were isolated from WT, $ROCK1^{+/-}$ and $ROCK2^{+/-}$ mice 24 h after MCAO occlusion after decapitation in deep isoflurane anesthesia. Brains were sliced in 2-mm sections and stained with 5 μmol/L DAF-FM diacetate (Invitrogen, CA, USA) for 20 min in the dark, quickly washed with PBS and observed with a Leica SP5 Confocal Microscope on 495/515 nm.

Assessment of NO Production

To measure NO_2, the stable breakdown product of NO in aqueous solutions, MHECs were cultured under standard conditions and starved for 12 h. Medium was then aspirated and replaced with 1 mL fresh serum-free DMEM. After 60 min, an aliquot was taken to measure background NO_2 production. Cultures were then treated with either Bradykinin (10^{-8} to 10^{-7} mol/L) or the calcium ionophore, A23187 (1×10^{-6} mol/L), for 60 min in fresh medium, after which a second aliquot was taken. NO_2 levels were measured using a Sievers NO analyzer (GE Analytical Instruments, CO, USA) and compared to freshly made standards prepared in DMEM.[S11]

Measurement of eNOS mRNA Stability

Using the RNA polymerase inhibitor, DRB, real-time RT-PCR was performed to determine the time-dependent effects of DRB (5×10^{-5} mol/L) on steady-state eNOS mRNA levels. The half-life of the eNOS mRNA was calculated by linear extrapolation. Each experiment was performed in triplicate and yielded similar results.[S5,S12] Total RNA was extracted from the PBS-perfused mouse aorta using TRIzol reagent. The transcriptome was reverse transcribed and a Quantitect SYBR Green RT-PCR kit (Qiagen) was used for amplification, using the 1-step protocol described by the manufacturer, and an ABI Prism 7900HT sequence detector (Applied Biosystems, CA, USA). The following primers were used to amplify partial cDNAs for eNOS and GAPDH: eNOS (forward, 5'-TTC CGG CTG CCA CCT GAT CCT AA-3', and reverse, 5'-AAC ATG TGT CCT TGC TCG AGG CA-3') and GAPDH (forward, 5'-GCA GTG GCA AAG TGG AGA TT-3', and reverse, 5'-CAC ATT GGG GGT AGG AAC AC-3'). Gene expression was measured using the $\Delta\Delta$Ct method. GAPDH was used as an endogenous control reference. Fold change in gene expression was measured relative to that in WT mice.

Immunoprecipitation

Bovine aortic EC (BAEC) lysate was incubated with eEF1A1 or ROCK2 antibody and protein G Sepharose (GE Healthcare Bio-Sciences) after 30-min stimulation with LPA (10 nmol/L) in 1% serum-containing DMEM. The immunoprecipitate was then subjected to immunoblotting with eEF1A1 and ROCK2 antibodies.

Phosphorylation Assay

Expression of GST-eEF1A1 proteins was induced with 0.1 mmol/L IPTG at 25°C for 5 h, and proteins were purified with glutathione agarose beads (GE Healthcare Bio-Sciences).[S1] Constitutively active ROCK1 and ROCK2 proteins were purchased from Millipore. Phosphorylation of GST-fusion proteins by ROCKs was performed according to the manufacturer's protocol, using [^{32}P]-γ-ATP (Perkin Elmer). After phosphorylation, proteins were separated on SDS-PAGE and the gel was dried, following

autoradiography.

RNA Electrophoretic Mobility Shift Assay (R-EMSA)

The 3'-UTR RNA of human eNOS was transcribed in vitro from 1 μg of linearized plasmid using T7 RNA polymerase and labeled with 50μCi [^{32}P]-UTP according to the manufacturer's instructions (Ambion). GST-fusion proteins were purified and dialyzed, and R-EMSA assays were performed, as described previously.[S1,S13]

Statistical Analysis

Band intensity on Western blots was quantified by densitometry using the Image-J software. All values are expressed as mean±SEM (standard error of the mean). All data were analyzed by 1- or 2-way ANOVA and Bonferroni test for post-hoc analyses. For comparisons of NDS, a non-parametric test was used (Mann-Whitney rank sum U-test). A value of $P<0.05$ was considered statistically significant.

References

S1. Yan G, You B, Chen SP, Liao JK, Sun J. Tumor necrosis factor-alpha downregulates endothelial nitric oxide synthase mRNA stability via translation elongation factor 1-alpha 1. *Circ Res* 2008; **103:** 591–597.

S2. Rikitake Y, Oyama N, Wang CY, Noma K, Satoh M, Kim HH, et al. Decreased perivascular fibrosis but not cardiac hypertrophy in ROCK1+/− haploinsufficient mice. *Circulation* 2005; **112:** 2959–2965.

S3. Okamoto R, Li Y, Noma K, Hiroi Y, Liu PY, Taniguchi M, et al. FHL2 prevents cardiac hypertrophy in mice with cardiac-specific deletion of ROCK2. *FASEB J* 2013; **27:** 1439–1449.

S4. Endres M, Laufs U, Huang Z, Nakamura T, Huang P, Moskowitz MA, et al. Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 1998; **95:** 8880–8885.

S5. Laufs U, Endres M, Stagliano N, Amin-Hanjani S, Chui DS, Yang SX, et al. Neuroprotection mediated by changes in the endothelial actin cytoskeleton. *J Clin Invest* 2000; **106:** 15–24.

S6. Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA. Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. *J Cereb Blood Flow Metab* 1997; **17:** 1143–1151.

S7. Betz AL, Iannotti F. Simultaneous determination of regional cerebral blood flow and blood--brain glucose transport kinetics in the gerbil. *J Cereb Blood Flow Metab* 1983; **3:** 193–199.

S8. Van Uitert RL, Levy DE. Regional brain blood flow in the conscious gerbil. *Stroke* 1978; **9:** 67–72.

S9. Noma K, Rikitake Y, Oyama N, Yan G, Alcaide P, Liu PY, et al. ROCK1 mediates leukocyte recruitment and neointima formation following vascular injury. *J Clin Invest* 2008; **118:** 1632–1644.

S10. Walsh JP, Caldwell KK, Majerus PW. Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol 4-phosphate. *Proc Natl Acad Sci USA* 1991; **88:** 9184–9187.

S11. Bauer PM, Fulton D, Boo YC, Sorescu GP, Kemp BE, Jo H, et al. Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine phosphorylation sites in endothelial nitric-oxide synthase. *J Biol Chem* 2003; **278:** 14841–14849.

S12. Laufs U, Liao JK. Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* 1998; **273:** 24266–24271.

S13. Hellman LM, Fried MG. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat Protoc* 2007; **2:** 1849–1861.