Redox Regulation of 14-3-3zeta Controls Monocyte Migration

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Running Title: 14-3-3zeta and Monocyte Migration

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Chemicals and Reagents

N-Ethylmaleimide (Cat# 04260), sodium ortho-vanadate (Cat# S6508), sodium fluoride (Cat# S7920), MG132 (Cat# C2211), reduced L-glutathione (Cat# G4251), and diamide (Cat# D3648) were obtained from Sigma (St. Louis, MO). Biotinylated glutathione ethyl ester (BioGEE) (Cat# G36000) and streptavidin-agarose (Cat# S951) were purchased from Invitrogen (Grand Island, NY). Complete Mini Protease Inhibitor Cocktail Tablets (Cat# 11836170001) were from Roche Diagnostics (Indianapolis, IN). Protein G Sepharose (Cat# 17-0618-01) was from GE Healthcare (Pittsburgh, PA). Z-VAD-FMK (Cat# FMK001) was from R&D Systems (Minneapolis, MN). Antibodies directed against phospho-cofilin (Cat# 3313), total cofilin (Cat# 3318), and the 14-3-3 Family Antibody Sampler Kit (Cat# 9769) were purchased from Cell Signaling (Danvers, MA). The antibody directed against SSH1L (Cat# A301-308A) was from Bethyl Laboratories (Montgomery, TX). The antibody directed against histidine tag (6xHis) (Cat# 37-2900) was from Life Technologies (Grand Island, NY). The antibody directed against glutathione (Cat# MAB5310) was from Millipore (Billerica, MA). Antibodies directed against LIMK1 (Cat# sc-8387) and actin (Cat# sc-1615) were purchased from Santa Cruz Biotechnologies (Dallas, TX), and the Antibodies directed against Flag (Cat# F1804), SSH2 (Cat# SAB1411536), and SSH3 (Cat# SAB1411021) were from Sigma.

Human Monocyte Culture

Human THP-1 monocytic cells (Cat# TIB-202, ATCC) were cultured in THP-1 medium: glucose-free RPMI-1640 (Cat# 10-043-CV, Cellgro, Manassas, VA) supplemented with 5 mmol/L D-glucose (Cat# G7021, Sigma), 2% Glutamax (Cat# 35050-061, Gibco, Grand Island, NY), 1% sodium pyruvate (Cat# 25-000-C1, Cellgro), 1% penicillin/streptomycin (Cat# 30-001-C1, Cellgro), 1% HEPES (Cat#15630-080, Gibco), 0.1% β-2-mercaptoethanol (Cat# M3148, Sigma) and supplemented with low-endotoxin 10% fetal bovine serum (FBS, Cat# 10082, Gibco). Cells were maintained at 37°C, in 5% CO_2, and 95% humidity. Metabolic stress was induced by incubating THP-1 monocytes for 24 h in THP-1 medium supplemented with 100 µg/ml LDL and 20 mmol/L D-glucose (LDL+HG). To stimulate cofilin phosphorylation and cell migration, cells activated with recombinant MCP-1 (2 nmol/L, Cat# 479-JE-010, R&D Systems) for the times indicated.

LDL Isolation

LDL was isolated by ultracentrifugation from pooled plasma from healthy blood donors as described previously [1].

Animals

Male LDL-$R^{-/-}$ mice (B6.129S7-$Ldlr^{tmlHer}$/J) were obtained from The Jackson Laboratories (Bar Harbor, ME) and housed in colony cages, maintained on a 12h light/12h dark cycle. Ten 18 week-old LDL-$R^{-/-}$ mice were randomized into 2 groups: low-fat diet (LFD) and high-fat diet (HFD). To induce hypercholesterolemia, mice were fed for 10 weeks HFD (Cat# AIN-76A, BioServ, Frenchtown, NJ) containing 21% fat (wt/wt) and 0.15% cholesterol (wt/wt). Grx1-deficient mice were a kind gift from Dr. Ye-Shih Ho, Wayne State University. All studies were performed with the approval of the UTHSCSA Institutional Animal Care and Use Committee.

Mouse Monocyte Isolation and Purification

Blood was drawn from the jugular vein of LFD and HFD-fed LDL-$R^{-/-}$ mice. Blood was treated with EasySep™ Red Blood Cell Lysis Buffer (Cat# 20110, Stem Cell Technologies, Vancouver, Canada), and monocytes were purified by negative selection using the EasySep™ mouse monocyte enrichment kit (Cat# 19761, Stem Cell Technologies), according to the manufacturer's instructions.

***In Vitro* Chemotaxis Assay**

Chemotaxis assays were conducted in 48-well modified Boyden chamber (Cat# AP48, NeuroProbe, Gaithersburg, MD) as described previously [2]. Briefly, cells were washed and resuspended (0.5-1 x 10^6/ml) in THP-1 cell culture medium (see above) supplemented with 0.1% FBS. Cells were then loaded into the upper wells of the Boyden chamber. The lower wells contained either vehicle or MCP-1 (2 nmol/L). A 5 μm polyvinyl pyrrolidone-free polycarbonate membrane (Cat# PFB5, NeuroProbe) was layered between the upper and lower chambers, and the chamber was incubated for 1.5 h at 37°C and 5% CO_2. The membrane was washed and cells removed from the upper side of the filter. Transmigrated cells were stained with Diff-Quik® Set (Cat# B4132-1A, Dade Behring, Deerfield, IL) and counted under a light microscope in four separate high power fields at 400X magnification.

14-3-3zeta Overexpression

Total RNA was isolated from human macrophages and applied as a template for cDNA synthesis with Revert Aid First Strand cDNA Synthesis Kit (Cat# K1622, Thermo Fisher Scientific, Pittsburgh, PA). To generate an N-terminal-Flag-tagged 14-3-3zeta, a DNA fragment containing full-length human 14-3-3zeta coding sequence (1–738bp) was generated by PCR amplification of human macrophage cDNA using the forward primer 5'-AAAAGGTACCATGGACTACAAGGACGACGATGACAAGGATAAAAATGAGCTGGTTC -3' and the backward primer 5′- TTTTGAATTCTTAATTTTCCCCTCCTTCTCCTGCTT -3′ followed by cloning into KpnI and EcoRI-restricted MCS of pcDNA3.1 (Cat# V79020, Invitrogen). To generate the C25S point mutant of 14-3-3 zeta, the "megaprimer" methods were performed [3]. For the C25S mutant megaprimer, forward primer of Flag-14-3-3zeta and backward primer 5′-TACAGACTTCATGCTGGCTGCCATGTCATC -3′ were used.

Lentiviral Transduction of THP-1 Monocytic Cells

pHIV-Flag-14-3-3zeta lentiviral plasmid was constructed by transferring the Flag-14-3-3zeta (WT or C25S) gene from pcDNA3.1-Flag-14-3-3zeta (WT or C25S) into pHIV-EGFP lentiviral plasmid (Addgene Plasmid # 21373, Cambridge, MA). All lentiviral supernatants were prepared by cotransfection of HEK-293T cells with one of the vector transfer constructs, envelope vector (pVSV-G), and the packaging vectors pMDLg/pRRE and RSV-Rev. The culture medium was replaced 12 h after transfection and viral supernatants were collected 48 h post-transfection. The viral supernatants were cleared by low-speed centrifugation, filtered through a 0.45 μm syringe filter and concentrated 100-fold using a Lenti-X™ Concentrator (Cat# 631231, Clontech, Mountain View, CA). Virus titers were determined by p24 ELISA following the manufacturer's instructions (Cat# 632200, Clontech). Viral pellets were resuspended in Opti-MEM (Cat# 31985062, Invitrogen) and stored at - 80°C. For THP-1 monocytes transduction, 2 × 10^6 cells suspended in 1 ml of THP-1 medium were plated in 12-well plates, and viruses (MOI = 10) were added to each well. The plate was centrifuged for 1 h at 800 × g at 25°C. THP-1 monocytes were incubated for 16 h. After sixteen hours, cells were washed and resuspended in THP-1 medium with 10% FBS.

14-3-3zeta Knockdown

Control and 14-3-3zeta-specific siRNA (sequence 1 or sequence 2) and control siRNA were purchased from Ambion (Grand Island, NY). Transfection was performed using the TransIT-X2 transfection reagent (Cat# MIR 6004, Mirus Bio, Madison, WI). THP-1 monocytes ($5x10^5$ per well) were seeded on a 12-well plate. For each well to be transfected, a transfection mixture was made by mixing of siRNA (25 nM) in 100µl Opti-MEM and adding 3µl of TransIT-X2 reagent. Following a 30 min incubation, the mixture was added to each well followed by a 72 h incubation at 37°C.

Phosphatase Activity Assay

To evaluate endogenous SSH1L phosphatase activity, SSH1L was immunoprecipitated from THP-1 monocytes incubated with either vehicle or LDL+HG for 24 h. Assays were initiated by adding 200 µmol/l of a phospho-cofilin 1 (Ser-3) peptide (Cat# CX1155, ECM Biosciences, Versailles, KY) as a substrate to immunoprecipitates in 20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1% NP-40 and warmed to 37°C [4]. The reaction was stopped after 15 min. The release of free phosphate was evaluated by its colorimetric reaction with malachite green (Cat# 20-105, Millipore), and the color intensity was measured in a 96-well plate using a VersaMax (Molecular Devices, Sunnyvale, CA) at 595 nm. Phosphate released by SSH1L was quantified from a standard curve prepared with known amounts of KH_2PO_4.

***In Vitro* Kinase Assay**

Cells were washed with phosphate-buffered saline, lysed in the lysis/kinase buffer (50 mmol/l Hepes, pH 7.4, 150 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l MnCl2, 10 mmol/l NaF, 1 mM Na_3VO_4, 5% glycerol, 1% Nonidet P-40) supplemented with protease inhibitor. After centrifugation, supernatants were precleared with protein G-Sepharose at 4 °C for 1 h, and incubated overnight at 4 °C with anti-LIMK1 antibody and protein G-Sepharose. The immunoprecipitates were washed twice with the lysis/kinase buffer. Immunoprecipitates were incubated for 20 min at 30°C in lysis/kinase containing 50 µmol/l ATP and substrate protein (His_6-cofilin 0.25 mg/ml) and then resolved by SDS-PAGE [5, 6]. The kinase activity was measured by Western blot analysis with anti-phospho-cofilin and anti-histidine tag antibodies.

Adenovirus-Mediated Expression of Glutaredoxin 1

To overexpress human glutaredoxin1 (Grx1), we used a previously described Dox-controlled Tet-On adenoviral system expressing a Grx1-EGFP fusion protein [2]. THP-1 monocytes were incubated for 24 h with adenoviruses (MOI = 25) in THP-1 medium supplemented with 10% FBS. Transgene expression was induced by adding Dox (1 µg/mL; Sigma) for 24 h.

Monocyte F-actin/G-actin Ratios

Ratios of filamentous and monomeric actin in THP-1 monocytes were determined as described previously [2]. THP-1 monocytes infected with a doxycycline (Dox)-inducible adenoviral vector carrying the sequence for a Grx1-EGFP fusion protein (pAd-Grx1) were pre-incubated with either vehicle or Dox (1 µg/mL; Sigma) for 24 h, and primed with LDL+HG. Cells were then stimulated for 30 min with either vehicle or MCP-1. Cells were lysed, homogenized and the total cellular actin pool was separated by ultracentrifugation into globular actin (G-actin) and filamentous actin (F-actin) using the G-actin/F-actin *in vivo* Assay Kit (Cytoskeleton). G-actin and F-actin fractions were then separated by SDS-PAGE and actin was quantified by Western blot analysis.

***In vitro S*-glutathionylation of 14-3-3zeta Protein**

Immunoprecipitated Flag-14-3-3zeta (WT or C25S) was *S*-glutathionylated by incubating the protein in PBS for 1 h at room temperature with 125 µmol/L glutathione (GSH) and 100 µmol/L

diamide. Following incubation, immunoprecipitates were washed three times with PBS to remove excess reagents.

Western Blot Analysis

Cells were washed with ice-cold PBS and lysed on ice in RIPA lysis buffer (50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with protease inhibitor and phosphatase inhibitors. Aliquots with equal amounts of protein were loaded and separated on a SDS-PAGE gel. Proteins were transferred to polyvinylidene difluoride membranes (Cat# ISEQ00010, Millipore) and probed using specific antibodies as indicated. Bands were detected by chemiluminescence on a KODAK Image Station 4000MM. To control for sample loading, blots were subsequently stripped and reprobed for total cofilin, Flag, or actin.

Detection of *S*-glutathionylated14-3-3zeta

THP-1 monocytes were pre-incubated for 1 h with culture medium containing 250 µmol/L BioGEE [7], and primed with LDL+HG. Cells were washed with ice-cold PBS and lysed in a RIPA buffer containing 10 mmol/L N-ethylmaleimide to block further thiol oxidation. Lysates (500 µg protein) were incubated for 1 h at 4°C with streptavidin-conjugated agarose beads. Beads were then rinsed three times with lysis buffer, *S*-glutathionylated proteins were released from the beads with 10 mmol/L dithiothreitol, separated by SDS-PAGE, and 14-3-3zeta levels were evaluated by Western blot analysis.

Immunohistochemistry

Serial cryosections of the aortic root from mice were double immunostained according to published protocols [8, 9]. After blocking for 1 h with 10% Normal Goat Serum (Cat# 71-00-27, KPL), sections were incubated overnight at 4°C humidified chamber with the following primary antibodies: Rat anti-mouse CD68: Biotin (1:200; Cat# FA-11, AbD Serotec), anti-phospho-cofilin Ab (1:100; Cat# 3313, Cell Signaling), anti-14-3-3zeta Ab (1:100; Cat# PA5-27317, Pierce), Sections were washed and primary antibodies were detected with simultaneously fluorescently labeled for 1 h at room temperature in a dark humidified chamber with Cy2 conjugated streptavidin secondary antibody (1:500), Cy3-Goat Anti-Rabbit (1:500) (Jackson ImmunoResearch) and trihydrochloride, trihydate (Hoechst, Cat#33342, Molecular Probes) to detect nuclei. The sections were mounted with FlourSave reagent (Cat# 345789, Calbiochem) and visualized under a fluorescent microscope using appropriate filters. Fluorescent images at 40X, 10X, and 4X magnification (Leica: DM1000 and Olympus Camera). Non-specific antibody reactions were assessed by omitting the primary antibody.

Statistics

Data were analyzed using ANOVA (Sigma Stat 12.0). Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed by using the Least Significant Difference method. All data are presented as mean ± SE of at least 3 independent experiments. Results were considered statistically significant at the $P<0.05$ level.

SUPPLEMENTAL SECTION - REFERENCES

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