ONLINE SUPPLEMENT

HISTONE DEACETYLASE (HDAC) 4 CONTROLS NEOINTIMAL HYPERPLASIA VIA STIMULATING PROLIFERATION AND MIGRATION OF VASCULAR SMOOTH MUSCLE CELLS

Short title: HDAC4 controls vascular remodeling

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Supplemental Materials and Methods

Animal care and treatment were conducted in conformity with institutional guidelines of The Kitasato University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal research was approved by ethical committee of School of Veterinary Medicine, The Kitasato University.

Materials

Reagent sources were as follows: platelet-derived growth factor (PDGF)-BB (PeproTech, Inc., Rocky Hill, NJ, USA); MC1568 (AdooQ Bio Science, Irvine, CA, USA). MC1568, (E)-3-(4-((E)-3-(3-fluorophenyl)-3-oxoprop-1-enyl)-1-methyl-1H-pyrrol-2-yl)-N-hydroxyacrylamide, is a novel selective class IIa histone deacetylases (HDAC)s inhibitor with IC50 of 220 nM 1; N-acetyl-L-cysteine (NAC) (Sigma Aldrich, St. Louis, MO, USA); KN93 (Wako, Osaka, Japan); SB203580 (Jena Bioscience Gmbh, Germany).

Antibody sources were as follows: phospho-p38 mitogen-activated protein kinase (MAPK) (Promega, Madison, WI, USA); total-p38MAPK and phospho- Ca2+ /calmodulin (CaM)-dependent protein kinase (CaMK)II (Thr286) (Santa Cruz Biotech, Santa Cruz, CA, USA); total-actin (Sigma Aldrich); HDAC4 and phospho-HDAC4 (Ser632) (Eno Gene, Nanjing, China); phospho-heat shock protein (HSP) 27 (Enzo Life Science, Plymouth Meeting, PA, USA); total-cyclin D1 (Gene Tex, Irvine, CA, USA); 4-hydroxy-2-nonenal (4-HNE) (Japan Institute for the Control of Aging, Shizuoka, Japan).

Culture of vascular smooth muscle cells (SMCs)

Male Wistar rats (7-9-week-old) were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguination. The superior mesenteric artery was isolated. SMCs isolated from mesenteric artery were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) 2. Passage 4 to 20 SMCs at 80 to 90 % confluence were growth arrested by incubating in DMEM containing 0% FBS for 24 h before stimulation.

Small interfering RNA (siRNA) transfection

One day after SMCs were subcultured, they (30-40% confluent) were transfected for 24 h with siRNA against HDAC4 (HDAC4 siRNA, UGAUAUGUUCAUGCAGCUGt) (Nippon EGT, Toyama, Japan) or non-silencing control siRNA (Qiagen, Valencia, CA, USA) using Lipofectamine 2000 (Invitrogen) dissolved in Optimem (Invitrogen) at a final concentration of 40 nM 3. And then, SMCs were recovered for additional 24 h before stimulation.

Cell proliferation analysis

Cell proliferation was examined by a cell counting using cell counting kit 8 (CC8; Dojindo, Kumamoto, Japan). After SMCs transfected with HDAC4 siRNA or control siRNA (40 nM, 24 h) in a 6-well
culture plate were stimulated with PDGF-BB (20 ng/ml) for 24 h, they were washed with Tris-Buffered Saline (TBS). And then, 25 μl of CC8 solution was added to each well and the plates were incubated for 1 h at 37 °C. Next, 90 μl of the CC8-containing medium was collected and 10 μl of 0.1 N HCl stop solution was added to terminate the reaction. An absorbance of the medium at 485 nm was read in a standard plate reader (Berthold Technologies, Tokyo, Japan). Cell proliferation was also examined by a bromodeoxyuridine (BrdU) incorporation assay kit (Exalpha Biologicals, Inc. Shirley, MA, USA). Briefly, the cells were seeded at a density of 2 × 10^3 cells/well in a 96-well culture plate. After transfected with HDAC4 siRNA or control siRNA (40 nM, 12 h), SMCs were treated with PDGF-BB (10 ng/ml, 24 h) in serum-free DMEM. The BrdU reagent was added to the wells for 12 h in the presence of PDGF-BB. After SMCs were washed several times with TBS, a fixative solution was added for 30 min. An anti-BrdU antibody was added for 30 min followed by the incubation with an anti-mouse IgG peroxidase-conjugate (1:2000) for 30 min. Tetra-methyl benzidine peroxidase substrate was then added for 30 min. An acid stop solution was added to terminate the reaction. An absorbance of the medium at 450 nm was read in a standard plate reader.

**Boyden chamber assay**

Boyden chamber assay was performed in Transwell chambers (Costar, Cambridge, MA, USA) as described previously 4. The polycarbonate membranes with an 8 μm pore were coated with 2% gelatin. After transfected with HDAC4 siRNA or control siRNA (40 nM, 24 h), SMCs were harvested using trypsin–EDTA and suspended in serum-free DMEM. A total of 600 μl serum-free DMEM was added in the lower chamber. The upper chamber was added with 5 × 10^4 cells in 100 μl media/well. PDGF-BB (10 ng/ml, 6 h) was added to the lower chamber. The membranes to which the cells migrated were fixed with methanol for 15 min and stained with Giemsa (Nacalai Tesque, Kyoto, Japan). After the membranes were washed with distilled water, non-migrated cells were wiped with cotton-swab. The number of migrated cells through the membranes was randomly counted in x100 fields under a light microscope (CKX31, Olympus, Tokyo, Japan) and averaged.

**Wound-induced migration assay**

After SMCs transfected with HDAC4 siRNA or control siRNA (40 nM, 24 h) in a 6-well culture dish were scratched in a cross shape by a 10 μl pipette tip, they were stimulated with DMEM with 2.5% FBS for 24 h. The images for wound healing were pictured in x100 fields under a light microscope (CKX31). The migrated length for cell was measured from the margin of wound width between 0 h and 24 h after stimulation.

**Western blotting**

Western blotting was performed as described previously 5, 6. Protein lysates were obtained by homogenizing SMCs with Triton-based lysis buffer (1% Triton X-100, 20 mM Tris, PH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na3VO4. 1 μg/ml leupeptin, and 0.1% protease inhibitor cocktail; Nacalai Tesque, Kyoto, Japan). Protein concentration was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Equal amounts of proteins
(8-10 µg) were separated by SDS-PAGE (7.5 or 10%), and transferred to a nitrocellulose membrane (Pall, Ann Arbor, MI, USA). After blocking with 3% bovine serum albumin (for phosphorylation-specific antibodies) or 0.5% skim milk (for others), the membranes were incubated with primary antibodies at 4 °C overnight, and then visualized using horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, 1 h) and the EZ-ECL system (Biological industries, Kibbutz Beit Hesmek, Israel). Equal loading of protein was confirmed by measuring total protein or actin expression. The results were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan).

**Measurement of reactive oxygen species (ROS) production**

Intracellular ROS production in SMCs was examined by a fluorescence staining using 2′, 7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA, Invitrogen). After treatment for 90 min with PDGF-BB in the presence of HDAC4 siRNA or control siRNA, SMCs were loaded with H$_2$DCFDA (10 µM) for 30 min. Fluorescence images were obtained using a fluorescence microscope (BX-51, Olympus) equipped with cooled CCD camera (MicroPublisher 5.0 RTV, Roper Japan, Tokyo, Japan). The Image J software was used for the quantitative analysis of the images.

**Lucigenin assay**

After treatment for 30 min with PDGF-BB in the presence of HDAC4 siRNA or control siRNA, total cell lysates were harvested. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity was determined by a lucigenin assay. The reaction carried out in total volume of 200 µl assay buffer containing 10 µM lucigenin, 500 µM NADPH, and 30 µg of cell lysates was measured by a TriStar LB941 lumino meter (Berthold, Bad, Wildbad, Germany). After the samples were well mixed, chemiluminescence was continuously measured for 180 min. Chemiluminescence of relative light units per second (RLU/s) was obtained every 10 s and the area under the curve (AUC) of RLU/s value was compared.

**Mice carotid ligation model**

Male BALB/c mice (25–28 g: 10-week-old) were underwent ligation of left carotid artery under the pentobarbital anesthesia (50 mg/kg) as described previously. This ligation model induces neointimal hyperplasia via proliferation and migration of SMCs due to cessation of blood flow. After the ventral surface of neck in the median line was incised, the right and left common carotid arteries were isolated. A 7-0 silk was passed under the left carotid just proximal to the bifurcation. And then, the artery was ligated. The right carotid artery was used as a sham control. MC1568 or vehicle (carboxymethylcellulose, CMC) was intraperitoneal administered at a dose of 50 mg/kg every other day for 3 weeks. After the mice were anesthetized with urethane (1.5 g/kg, i.p.) and euthanized by exsanguination, the carotid arteries were isolated. After the fat and connective tissues were removed, the samples were used for the histological and immunohistochemical examinations.
Histological and immunohistochemical examinations

The arterial samples were fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin. The thin sections (4 µm-thick) were stained with hematoxylin and eosin as described previously 6. Immunohistochemical staining for phospho-HDAC4, phospho-p38MAPK, phospho-HSP27 or 4-HNE was performed by a peroxidase staining kit (LSAB2; Dako, Glostrup, Denmark). Rabbit polyclonal antibodies against phospho-HDAC4, phospho-p38MAPK, phospho-HSP27 and 4-HNE were used as the primary antibody. The images were obtained using a light microscope (BX-51). Intima/media ratio was calculated by using Image J software.

Statistical Analysis

Data are shown as means ± SEM. Statistical evaluations were performed using one-way ANOVA followed by Bonferroni’s test for comparisons in more than three groups and by Student's t-test between two groups. Values of *P* < 0.05 were considered statistically significant.
References


Figure S1. Effects of histone deacetylase (HDAC) 4 knockdown on fetal bovine serum (FBS)-induced smooth muscle cells (SMCs) migration. SMCs migration was determined by a wound-induced migration assay. (A) Representative photomicrographs of SMCs transfected with HDAC4-specific small interfering RNA (siRNA) (HDAC4 siRNA) or non-silencing control siRNA before and after 2.5% FBS stimulation for 24 h were shown. Scale bar: 50 μm. (B) The migrated length of cells was shown as fold increase relative to control siRNA without FBS stimulation (n=6).

**P<0.01 vs. control siRNA without FBS; #P<0.05 vs. cont siRNA+FBS.
**Figure S2.** Effects of HDAC4 knockdown on platelet-derived growth factor (PDGF)-BB-induced cytoskeletal reorganization in SMCs. Actin cytoskeleton was examined by a rhodamine phalloidin staining. Representative photomicrographs of SMCs transfected with HDAC4 siRNA or control siRNA before PDGF-BB (10 ng/ml, 6 h) stimulation were shown (n=3-5). Arrows show the lamellipodia formation. Scale bar: 50 μm.
Figure S3. Time- and concentration-dependent effects of PDGF-BB stimulation on expression and phosphorylation of HDAC4 in SMCs. After SMCs were treated with 20 ng/ml PDGF-BB for 6 h-36 h (A) or with 1-20 ng/ml PDGF-BB for 24 h (B), total cell lysates were harvested. Expression of HDAC4 protein was determined by Western blotting and shown as fold increase relative to control (A, n=4, B, n=4-8). After SMCs were treated with 10 ng/ml PDGF-BB for 1-60 min (C) or with 1-20 ng/ml PDGF-BB for 30 min (D), total cell lysates were harvested. Phosphorylation of HDAC4 (at Ser632) was determined by Western blotting and shown as fold increase relative to control (C, n=4, D, n=4-8). *P<0.05, **P<0.01 vs. cont.
Figure S4. Effect of an antioxidant drug, N-acetyl-L-cysteine (NAC) (A) or a p38 inhibitor, SB203580 (B) on PDGF-BB-induced SMCs proliferation. After pretreated with NAC (3 mM, 30 min) or SB203580 (3, 10 μM, 30 min), SMCs were stimulated with PDGF-BB (20 ng/ml, 24 h). Cell proliferation was evaluated by a cell counting. The cell number (A, n=3-4, B, n=3) was shown as fold increase relative to control. *P<0.05, **P<0.01 vs. cont; #P<0.05, ##P<0.01 vs. PDGF-BB.
Effects of PDGF-BB stimulation on Ca\(^{2+}\)/Calmodulin (CaM)-dependent protein kinase (CaMK)II activity in SMCs (A). SMCs were treated with 10 ng/ml PDGF-BB for varying time (30 sec-5 min), phosphorylation of CaMKII (at Thr286) was determined by Western blotting (n=6-7). Equal protein loading was confirmed using total actin antibody.

Effects of a CaMKII inhibitor, KN93 on PDGF-BB-induced HDAC4 activity (B). After SMCs were treated with 10 ng/ml PDGF-BB for 30 min in the absence or presence of KN93 (10 \( \mu \)M, pretreatment for 30 min), phosphorylation of HDAC4 (n=4) was determined by Western blotting and shown as fold increase relative to control. Equal protein loading was confirmed using total actin antibody. Effects of KN93 on PDGF-BB-induced SMCs proliferation (C). After pretreated with KN93 (3, 10 \( \mu \)M, 30 min), SMCs were stimulated with PDGF-BB (20 ng/ml, 24 h). Cell proliferation was evaluated by a cell counting assay. The cell number was shown as fold increase relative to control. Effects of KN93 on PDGF-BB-induced SMCs migration (D). After pretreated with KN93 (10 \( \mu \)M, 2 h), SMCs were stimulated with PDGF-BB (10 ng/ml, 6 h). Migration of SMCs was determined by a Boyden chamber assay. The number of migrated cell was shown as fold increase relative to control (n = 3). **P<0.01 vs. cont; #P<0.05 , ##P<0.01 vs. PDGF-BB.
Figure S6. Effects of long-term treatment with a class IIa HDACs inhibitor, MC1568 on carotid reactive oxygen species (ROS) production in mice ligation model. After MC1568 was intraperitoneally administered to mice (10-week-old) at a dose of 50 mg/kg every other day for 3 weeks (n=4), carotid artery was harvested. The thin paraffin sections (4 mm) were immunohistochemically stained with antibody to 4-Hydroxy-2-nonenal, a ROS marker. Representative photomicrographs were shown. Neointima (N), media (M) and adventitia (A) were shown. Scale bar: 50 μm.
Figure S7. Effects of long-term MC1568 treatment on carotid activation of p38 mitogen-activated protein kinase (MAPK) and heat shock protein (HSP) 27 in mice ligation model. After MC1568 was intraperitoneally administered to mice (10-week-old) at a dose of 50 mg/kg every other day for 3 weeks (n=4), carotid artery was harvested. The paraffin sections (4 mm) were immunohistochemically stained with antibody to phospho-p38 or HSP27. Representative photomicrographs were shown. Neointima (N), media (M) and adventitia (A) were shown. Scale bar: 50 μm.
Figure S8. Effects of MC1568 on PDGF-BB-induced SMCs proliferation, migration and cellular signals. After pretreated with MC1568 (5 μM, 30 min), SMCs were stimulated with PDGF-BB (20 ng/ml, 24 h) (A). Cell proliferation was evaluated by a cell counting assay (n=6). The cell number was shown as fold increase relative to control. After pretreated with MC1568 (5 μM, 2 h), SMCs were stimulated with PDGF-BB (10 ng/ml, 6 h) (B). SMCs migration was determined by a Boyden chamber assay. The number of migrated cell was shown as fold increase relative to control (n =4). Effects of MC1568 on PDGF-BB-induced activation of HDAC4 (C), p38MAPK (D) and HSP27 (E). After SMCs were treated with 10 ng/ml PDGF-BB for 30 min in the absence or presence of MC1568 (5 μM, pretreatment for 30 min), phosphorylation of HDAC4 (n=4), p38MAPK (n=4) and HSP27 (n=4) was determined by Western blotting and shown as fold increase relative to control. Equal protein loading was confirmed using total antibody or total actin antibody. **P<0.01 vs. cont; #P<0.05, ##P<0.01 vs. PDGF-BB.
Figure S9. Summary of the present results. PDGF-BB-induced activation of HDAC4 mediates ROS-dependent proliferation and migration of vascular SMCs via activation of p38MAPK and HSP27 in a CaMKII-dependent manner, which may lead to vascular hypertrophy.