Histone Deacetylase 4 Controls Neointimal Hyperplasia via Stimulating Proliferation and Migration of Vascular Smooth Muscle Cells

Tatsuya Usui, Tomoka Morita, Muneyoshi Okada, Hideyuki Yamawaki

Abstract—Histone deacetylases (HDACs) are transcriptional coregulators. Recently, we demonstrated that HDAC4, one of class IIa family members, promotes reactive oxygen species–dependent vascular smooth muscle inflammation and mediates development of hypertension in spontaneously hypertensive rats. Pathogenesis of hypertension is, in part, modulated by vascular structural remodeling via proliferation and migration of vascular smooth muscle cells (SMCs). Thus, we examined whether HDAC4 controls SMC proliferation and migration. In rat mesenteric arterial SMCs, small interfering RNA against HDAC4 inhibited platelet-derived growth factor (PDGF)-BB–induced SMC proliferation as determined by a cell counting and bromodeoxyuridine incorporation assay as well as migration as determined by Boyden chamber assay. Expression and activity of HDAC4 were increased by PDGF-BB. HDAC4 small interfering RNA inhibited phosphorylation of p38 mitogen–activated protein kinase and heat shock protein 27 and expression of cyclin D1 as measured by Western blotting. HDAC4 small interfering RNA also inhibited PDGF-BB–induced reactive oxygen species production as measured fluorometrically using 2′, 7′-dichlorofluorescein diacetate and nicotinamide adenine dinucleotide phosphate oxidase activity as measured by lucigenin assay. A Ca2+/calmodulin-dependent protein kinase II inhibitor, KN93, inhibited PDGF-BB–induced SMC proliferation and migration as well as phosphorylation of HDAC4. In vivo, a class IIa HDACs inhibitor, MC1568 prevented neointimal hyperplasia in mice carotid ligation model. MC1568 also prevented increased activation of HDAC4 in the neointimal lesions. The present results for the first time demonstrate that HDAC4 controls PDGF-BB–induced SMC proliferation and migration through activation of p38 mitogen–activated protein kinase/heat shock protein 27 signals via reactive oxygen species generation in a Ca2+/calmodulin-dependent protein kinase–dependent manner, which may lead to the neointimal hyperplasia in vivo. (Hypertension. 2014;63:397-403.)

Key Words: hypertension ◼ intercellular signaling peptides and proteins ◼ muscle, smooth ◼ reactive oxygen species ◼ signal transduction

Histone deacetylases (HDACs) play a central role in the epigenetic regulation of gene expression. To date, 18 human HDACs have been identified, and they were classified into 4 classes: class I HDACs (HDAC1, 2, 3, and 8), class II HDACs (HDAC4, 5, 6, 7, 9, and 10), class III HDAC (Sir2), and class IV HDAC (HDAC11). Class II HDACs are further classified into class Ila (HDAC4, 5, 7, 9, and the HDAC9 splice variant myo- cyte enhancer factor [MEF]-2 interacting transcription repressor) and class Iib (HDAC6 and 10).1 Class Ila HDACs (4, 5, 7, and 9) seem to have critical roles in many diseases processes, including cardiac diseases,2 cancer,3 and viral infection.4 Among them, recent study demonstrated that HDAC4 plays important roles in mediating cardiovascular diseases. For example, (1) Ca2+/calmodulin-dependent protein kinase (CaMK) II promoted hypertrophic growth via phosphorylation of HDAC4 in cultured cardiomyocytes,5 (2) activation of HDAC4 promoted angiogenesis II–induced vascular smooth muscle hypertrophy,6 and (3) CaMKIIα mediated cardiac hypertrophy by interfering with the HDAC4-MEF-2 signaling pathway.7 In addition, we have recently demonstrated that expression of HDAC4 protein increased in mesenteric artery of spontaneously hypertensive rats.8 Moreover, we demonstrated that HDAC4 promoted reactive oxygen species (ROS)-dependent vascular inflammation and mediated the development of hypertension in spontaneously hypertensive rats.9

Proliferation and migration of vascular smooth muscle cells (SMCs) lead to the medial thickening (structural remodeling), which has a significant role on the processes of hypertension development.10,11 In addition, ROS contribute to the pathogenesis of cardiovascular diseases, including hypertension12 via promoting proliferation and migration of vascular SMCs.13,14 Nevertheless, it remains to be clarified how HDAC4 controls SMCs proliferation and migration through ROS regulation. Therefore, we examined whether HDAC4 affects vascular neointimal hyperplasia via SMC proliferation and migration by especially focusing on cellular signaling related to ROS.
Here, for the first time, we demonstrate that HDAC4 mediates ROS-dependent SMC proliferation and migration via activation of p38 mitogen–activated protein kinase (MAPK)/heat shock protein (HSP) 27 pathway in a CaMKII-dependent manner, which may lead to the in vivo neointimal hyperplasia.

**Materials and Methods**
The detailed methods are available as an online-only Data Supplement.

**Results**

**Effects of HDAC4 Knockdown on Platelet-Derived Growth Factor-BB–Induced SMC Proliferation**
First, we examined whether HDAC4 mediates SMC proliferation. SMC proliferation was evaluated by a cell counting. Platelet-derived growth factor (PDGF)-BB (20 ng/mL, 24 hours)–induced SMC proliferation was significantly inhibited by HDAC4 small interfering RNA (siRNA; Figure 1A and 1B). SMC proliferation was also evaluated by a bromodeoxyuridine incorporation assay. PDGF-BB (10 ng/mL, 24 hours)–induced bromodeoxyuridine incorporation was significantly inhibited by HDAC4 siRNA (Figure 1C). We confirmed that PDGF-BB (20 ng/mL, 24 hours)–induced expression of HDAC4 was significantly inhibited by HDAC4 siRNA (Figure 1D).

**Effects of HDAC4 Knockdown on PDGF-BB–Induced SMC Migration**
To clarify the effects of HDAC4 knockdown on SMC migration, we used a Boyden chamber assay. PDGF-BB (10 ng/mL, 6 hours)–induced SMC migration was significantly inhibited by HDAC4 siRNA (Figure 2A and 2B). To verify the effects of HDAC4 knockdown on SMC migration by another type of assay, we used a wound-induced migration assay. Fetal bovine serum (2.5%, 24 hours)–induced SMC migration was significantly inhibited by HDAC4 siRNA (Figure S1A and S1B in the online-only Data Supplement).

**Effects of HDAC4 Knockdown on PDGF-BB–Induced Cytoskeletal Reorganization in SMCs**
Cytoskeletal reorganization is essential for SMC migration.15 Thus, we examined the effects of HDAC4 knockdown on actin cytoskeletal reorganization in SMCs by a phalloidin staining. HDAC4 siRNA prevented lamellipodia formation induced by PDGF-BB (10 ng/mL, 90 minutes; Figure S2).

**Effects of HDAC4 Knockdown on PDGF-BB–Induced Cellular Signals Related to Proliferation and Migration of SMCs**
Next, we examined whether HDAC4 mediates the proliferation/migration-related signals in SMCs. We first examined the effects of PDGF-BB stimulation on expression and activity of HDAC4. It was shown that PDGF-BB increased expression and phosphorylation of HDAC4 in a time- and concentration-dependent manner (Figure S3A–S3D). We further confirmed that PDGF-BB (10 ng/mL, 30 minutes)–induced phosphorylation of HDAC4 was significantly inhibited by HDAC4 siRNA (Figure 3A). PDGF-BB (10 ng/mL, 30 minutes)–induced SMC proliferation was significantly inhibited by HDAC4 siRNA (Figure 3B). The cell number was shown as fold increase relative to control siRNA without PDGF-BB (n=6–7).

**Figure 1.** Effects of histone deacetylase (HDAC) 4 knockdown on platelet-derived growth factor (PDGF)-BB–induced smooth muscle cell (SMC) proliferation. A, Representative photomicrographs of SMCs transfected with HDAC4-specific small interfering RNA (HDAC4 siRNA) or nonsilencing control siRNA (40 nmol/L, 24 hours) before PDGF-BB (20 ng/mL, 24 hours) stimulation were shown. Cell proliferation was evaluated by a cell counting assay. Scale bar, 50 μm. B, The cell number was shown as fold increase relative to control siRNA without PDGF-BB (n=6–7). C, Effects of HDAC4 knockdown on PDGF-BB–induced bromodeoxyuridine (BrdU) incorporation in SMCs. After SMCs were transfected with HDAC4 siRNA or control siRNA, they were stimulated with PDGF-BB (20 ng/mL, 24 hours). BrdU reagent was added to the cells for 12 hours in the presence of PDGF. The incorporated BrdU detected by an immunostaining using anti-BrdU antibody was shown as fold increase relative to control siRNA without PDGF-BB (n=6–8). D, Effects of HDAC4 knockdown on PDGF-BB–induced HDAC4 protein expression. After SMCs were transfected with HDAC4 siRNA or control siRNA, they were treated with PDGF-BB (20 ng/mL, 24 hours). HDAC4 expression (n=6–7) was determined by Western blotting and shown as fold increase relative to control siRNA without PDGF-BB stimulation. Equal protein loading was confirmed using total actin antibody. **P<0.01 vs control siRNA without PDGF-BB; #P<0.05, ###P<0.01 vs control siRNA+PDGF-BB.
30 minutes)–induced phosphorylation of p38MAPK (Figure 3C) was significantly inhibited by HDAC4 siRNA. HDAC4 siRNA had no effect on PDGF-BB–induced phosphorylation of extracellular signal-regulated kinase, c-jun N-terminal kinase, and Akt in SMCs (data not shown, n=5), suggesting the specificity of HDAC4 to p38MAPK/HSP27 signals. We confirmed that HDAC4 siRNA alone had no influence on the signals. We further examined the effects of HDAC4 knockdown on expression of PDGF-induced cell cycle regulatory protein, cyclin D1. Cyclin D1 is known to be required for progression of the G1 phase and plays a role for proliferative signals in G1.16,17 HDAC4 siRNA significantly inhibited PDGF (20 ng/mL, 24 hours)–induced expression of cyclin D1 (Figure 3D).

To further investigate the upstream mechanisms, we examined whether HDAC4 knockdown prevents PDGF-BB–induced ROS production in SMCs. PDGF-BB (10 ng/mL, 90 minutes) increased a 2′, 7′-dichlorodihydrofluorescein diacetate–sensitive fluorescent intensity compared with nonstimulated control (Figure 4A). HDAC4 siRNA significantly inhibited the PDGF-BB–induced ROS production (Figure 4A). It was reported in SMCs that PDGF-BB induced ROS production via activation of nicotinamide adenine dinucleotide phosphate oxidase (NOX).18,19 Then, we examined whether HDAC4 knockdown prevents PDGF-BB–induced NOX activity in SMCs. HDAC4 siRNA significantly inhibited PDGF-BB (10 ng/mL, 30 minutes)–induced NOX activity (Figure 4B).

Effects of an Antioxidant Drug, N-acetyl-L-Cysteine, or a p38MAPK Inhibitor, SB203580, on PDGF-BB–Induced SMC Proliferation
To further verify whether PDGF-BB–induced ROS generation or activation of p38MAPK signal mediates SMC proliferation, we pretreated SMCs with N-acetyl-L-Cysteine (NAC; 3 mmol/L, 30 minutes) or SB203580 (3, 10 μmol/L, 30 minutes) before PDGF-BB stimulation (20 ng/mL, 24 hours). NAC (Figure S4A) or SB203580 (Figure S4B) significantly inhibited PDGF-BB–induced SMCs proliferation. We previously confirmed that NAC or SB203580 significantly inhibited PDGF-BB–induced SMC migration as determined by a Boyden chamber assay.20

Effects of a CaMKII Inhibitor, KN93, on PDGF-BB–Induced SMC Proliferation and Migration
HDAC4 activity is regulated by CaMKII in various types of cells.6,7 Thus, we investigated whether CaMKII regulates PDGF-BB–induced proliferation and migration of SMCs. We confirmed that phosphorylation of CaMKII at Thr286 (Figure S5A) was significantly increased by PDGF-BB (10 ng/mL) at 1 minute, which corresponded to the previously published results by others.21 It was further confirmed that PDGF-BB (10 ng/mL, 30 minutes)–induced phosphorylation of HDAC4 (Figure S5B) was significantly inhibited by a CaMKII inhibitor, KN93 (10 μmol/L). PDGF-BB (20 ng/mL, 24 hours or 10 ng/mL, 6 hours)–induced SMC proliferation (Figure S5C) or migration (Figure S5D) was significantly inhibited by KN93 (3–10 μmol/L).

Effects of a Class IIa HDACs Inhibitor, MC1568, on Neointimal Hyperplasia in Mice Carotid Ligation Model
Because previous reports showed that PDGF signals regulate neointimal hyperplasia,22,23 we finally examined the effects of long-term MC1568 treatment (for 3 weeks) on neointimal hyperplasia in mice carotid ligation model. The neointimal lesions increased in the ligated left carotid arteries compared with sham control (right carotid arteries; Figure 5A and 5B).
MC1568 (50 mg/kg per day) significantly prevented the development of neointimal hyperplasia in the ligated artery (Figure 5A and 5B). To further examine whether HDAC4 controls the development of neointimal lesions, the effects of MC1568 on HDAC4 activity were examined in the neointimal lesions. MC1568 prevented the increased phosphorylation of HDAC4 in the neointimal lesions (Figure 6).

We further examined the involvement of ROS and p38MAPK/HSP27 signals in the neointimal formation. It was found that ROS and p38MAPK/HSP27 signals actually increased in the ligated arteries, which was prevented by MC1568 (Figure S6 and S7). We confirmed that MC1568 significantly inhibited the proliferation and migration as well as phosphorylation of HDAC4, p38MAPK, and HSP27 induced by PDGF-BB (Figure S8A–S8E).

Discussion

In the present study, we examined whether HDAC4 mediates vascular SMC proliferation and migration. The major findings of the present study are that inhibition of HDAC4 prevented PDGF-BB–induced proliferation, migration, activation of p38MAPK and HSP27, and ROS production in cultured vascular SMCs (Figures 1–4 and Figure S1). It was also found that an inhibitor of CaMKII prevented PDGF-BB–induced proliferation and migration as well as activation of HDAC4 in SMCs (Figure S5). In addition, we showed that an antioxidant drug, NAC, and a p38MAPK inhibitor, SB203580, inhibited PDGF-BB–induced proliferation in SMCs (Figure S4). In the previous study, we showed that NAC and SB20580 inhibited PDGF-induced migration of SMCs.20 It was also shown that an inhibitor of class IIa HDACs, including HDAC4, prevented neointimal hyperplasia in mice carotid ligation model. Collectively, our results indicate that PDGF-BB activates HDAC4 in a CaMKII-dependent manner, which leads to promotion of ROS-dependent SMC proliferation and migration via activation of p38MAPK/HSP27 signals in vascular SMCs (Figure S9). It is suggested that HDAC4 might be at least partly responsible for the neointimal hyperplasia in mice carotid ligation model through the stimulation of SMC proliferation and migration.

HDAC4 is a substrate for activated CaMKII.5,24,25 Recent study suggested that CaMKII δC moved HDAC4 to the cytosol, thereby stimulating nuclear MEF2 activity in vascular SMCs.26 In addition, it was reported that PDGF-BB induced HDAC4 phosphorylation and cytoplasmic sequestration, which promoted MEF2 activation and c-Jun expression in cultured SMCs.27 Similarly, CaMKII mediated angiotensin II–induced vascular SMCs hypertrophy through phosphorylation of
HDAC and subsequent MEF2 activation. Furthermore, it was reported that MEF2 mediated vascular inflammation via p38MAPK-dependent pathway. In this study, we demonstrated that HDAC4 gene knockdown inhibited PDGF-BB–induced phosphorylation of HDAC4 as well as p38MAPK and HSP27 (Figure 3). It was also shown that a CaMKII inhibitor, KN93, inhibited PDGF-BB–induced SMC proliferation and migration as well as phosphorylation of HDAC4 (Figure S5). These results imply that HDAC4 mediates PDGF-BB–induced SMC proliferation and migration through the activation of p38MAPK and HSP27 via MEF2 regulation. In addition, although the role of HDAC4 in cytoplasm after PDGF stimulation still remains to be clarified, it is presumed that the effects of HDAC4 siRNA might be mediated at least in part via decrease of cytoplasmic HDAC4.

Formation of actin-rich protrusions such as lamellipodia is an important process for cell migration. In addition, distribution of p38MAPK and HSP27 in lamellipodia seems to play an important role for the formation of lamellipodia in SMCs. It was reported that PDGF-BB–induced phosphorylation of p38MAPK was transiently observed at the leading edge of lamellipodia, and that phosphorylated p38MAPK remained at the base, whereas phosphorylated HSP27 was distributed only at the base of lamellipodia. It was indicated that the nonphosphorylated HSP27 at the leading edge displays actin-capping activity, whereas phosphorylated HSP27 might stabilize the actin network at the base, suggesting that HSP27 plays a role in the spatial organization of lamellipodia. In this study, we demonstrated that HDAC4 siRNA reduced PDGF-BB–induced phosphorylation of p38MAPK and HSP27 (Figure 3) as well as lamellipodia formation (Figure S2). It is of interest to investigate how HDAC4 controls distribution of phosphorylated and nonphosphorylated p38MAPK and HSP27 in the lamellipodium of SMCs.

In this study, we showed that HDAC4 knockdown inhibited PDGF-BB–induced ROS production and NOX activity in

Figure 4. Effects of histone deacetylase (HDAC) 4 knockdown on platelet-derived growth factor (PDGF)-BB–induced reactive oxygen species (ROS) production. A, ROS production was determined by a fluorescence staining using 2′, 7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA). After smooth muscle cells (SMCs) were treated with 10 ng/mL PDGF-BB for 90 minutes in the presence of control or HDAC4 siRNA (n=3–5), they were loaded with H$_2$DCFDA (10 μmol/L) for 30 minutes. Images were obtained using a fluorescence microscope. Fluorescent intensity was measured using Image J software and shown as fold increase relative to control siRNA without PDGF-BB stimulation. B, Effects of HDAC4 knockdown on PDGF-BB–induced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity. After SMCs were treated with 10 ng/mL PDGF-BB for 30 minutes in the presence of control or HDAC4 siRNA (n=4), total cell lysates were harvested. NOX activity was determined by lucigenin assay. The reaction performed in total volume of 200 μL assay buffer containing lucigenin, NADPH, and total cell lysates was measured by a luminometer. NOX activity stands for the area under the curve of chemiluminescence of relative light units per second value and was shown as fold increase relative to control siRNA without PDGF-BB stimulation. *P<0.05, **P<0.01 vs control siRNA without PDGF-BB; #P<0.05 vs control siRNA+PDGF-BB.

Figure 5. Effects of a class IIa histone deacetylases (HDACs) inhibitor, MC1568, on neointimal hyperplasia in mice carotid ligation model. MC1568 was intraperitoneally administered to mice (10 week old) at a dose of 50 mg/kg every other day (n=6). After 3 weeks, carotid arteries were harvested. The paraffin sections (4 μm) were made and stained with hematoxylin and eosin. Representative photomicrographs are shown in A. The ratio of neointimal to medial area was measured by Image J software (B, n=6). *P<0.05 vs ligation. Arterial neointima (N), media (M), and adventitia (A) are shown. Scale bar, 50 μm.
SMCs (Figure 4). Nevertheless, it remained to be clarified how HDAC4 controls NOX activity in SMCs. Because it was previously reported that HDACs inhibitor decreased gp91 phox (a component of NOX) expression in the left ventricle tissues from spontaneously hypertensive rats, it is presumed that HDAC4 might regulate NOX activity through induction of gp91 phox expression in SMCs. Furthermore, it was reported that PDGF-BB stimulated ROS production via p47 phox or Rac-1 activation (components of NOX) in vascular SMCs. Therefore, it is also considerable that HDAC4 might regulate NOX activity via p47 phox or Rac-1 activation.

It was recently reported that an inhibitor of both class I and II HDACs, trichostatin A, inhibited neointimal hyperplasia in a balloon injury model of rat carotid artery, suggesting that class I and class II HDACs at least in part mediate neointimal hyperplasia. Nevertheless, it remained to be clarified whether a specific inhibition of class IIa HDACs, including HDAC4, prevents neointimal hyperplasia. It was reported that MC1568 selectively inhibited activity of class IIa HDACs (IC50=220 nmol/L). It was shown in human breast cancer cells that MC1568 (5 μmol/L) selectively inhibited activity of HDAC4 but not HDAC1, one of class I HDACs members. In addition, it was reported that activity of HDAC4 and HDAC5 but not HDAC3 was inhibited in skeletal muscle and heart from the mice treated with MC1568 (50 mg/kg). In this study, we showed that HDAC4 mediates proliferation and migration of SMCs in vitro (Figures 1–4). We also showed for the first time that MC1568 (50 mg/kg) inhibited neointimal hyperplasia in vivo (Figures 5 and 6). These results suggest that HDAC4 might at least partly play a critical role for vascular neointimal hyperplasia in vivo. The neointimal hyperplasia is one of the major processes for hypertension development. In addition, there are several reports including our own showing that trichostatin A or valproic acid, which inhibits HDACs including class II HDACs, prevented the development of hypertension in rats. Thus, it is likely that MC1568 may reduce blood pressure in hypertensive animals.

**Perspectives**

For the first time, we demonstrate that HDAC4 controls ROS-dependent proliferation and migration of vascular SMCs. It was also suggested that HDAC4 may be at least partly responsible for the neointimal hyperplasia in mice carotid ligation model. Because migration and proliferation are important processes for the development of hypertension, further studies on HDAC4 might contribute to develop new pharmaceutical therapy for the prevention of hypertensive cardiovascular diseases.

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**Disclosures**

None.

**References**

What Is New?

- Histone deacetylases (HDACs) play roles on epigenetic regulation of gene expression.
- For the first time, we revealed in vascular smooth muscle cells that HDAC4 mediates platelet-derived growth factor-BB–induced proliferation and migration via reactive oxygen species–dependent mechanisms.
- It was also demonstrated that HDAC4 may be at least partly responsible for vascular remodeling after carotid artery ligation.

What Is Relevant?

- Vascular remodeling is a hallmark of hypertension, atherosclerosis, and restenosis after angioplasty.
- HDAC4 may at least partly play a pivotal role for vascular remodeling through the control of smooth muscle cell proliferation and migration.
- Therefore, further studies on HDAC4 might contribute to develop new pharmaceutical therapy for the prevention of hypertensive cardiovascular diseases.

Summary

HDAC4 controls platelet-derived growth factor-BB–induced smooth muscle cell proliferation and migration through activation of p38 mitogen–activated protein kinase/heat shock protein 27 signals via reactive oxygen species generation in a Ca2+/calmodulin-dependent protein kinase–dependent manner, which may lead to the vascular remodeling in vivo.
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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 IC$_{50}$ of 220 nM; 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- Ca$^{2+}$/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, UGAUAUGUUCAUGCGCUGt) (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 $2 \times 10^3$ 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. 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 $5 \times 10^4$ 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. 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 Na$_3$VO$_4$, 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 (H2DCFDA, Invitrogen) 2, 7. After treatment for 90 min with PDGF-BB in the presence of HDAC4 siRNA or control siRNA, SMCs were loaded with H2DCFDA (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 8. 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 luminometer (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 9. 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 intraperitoneally 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. 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.


**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.
Figure S5. 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.