Ubiquitin Carboxyl-Terminal Hydrolase L1, a Novel Deubiquitinating Enzyme in the Vasculature, Attenuates NF-κB Activation

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Objective—We identified a ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) gene, which encodes a deubiquitinating enzyme and is expressed in the vasculature, by functional screening of a human endothelial cell (EC) cDNA library. UCHL1 is expressed in neurons, and abnormalities in UCHL1 are responsible for inherited Parkinson’s disease via its effects on the ubiquitin-proteasome system. Therefore, the goal of present study was to clarify the role of the UCHL1 gene in vascular remodeling by evaluating nuclear factor-κB (NF-κB) inactivation in ECs and vascular smooth muscle cells (VSMCs).

Methods and Results—From Northern blot and immunohistochemical analysis, the UCHL1 gene was endogenously expressed in vascular ECs, VSMCs, and brain tissue. Expression of UCHL1 was markedly increased in the neointima of the balloon-injured carotid artery and was also present in atherosclerotic lesions from human carotid arteries. Overexpression of the UCHL1 gene significantly attenuated tumor necrosis factor (TNF)-α–induced NF-κB activity in vascular cells and increased inhibitor of kappa B-α (IκB-α), possibly through the attenuation of IκB-α ubiquitination, leading to decreased neointima in the balloon-injured artery. In contrast, knockdown of UCHL1 by small interfering RNA resulted in increased NF-κB activity in VSMCs.

Conclusions—These data suggest that UCHL1 may partially attenuate vascular remodeling through inhibition of NF-κB activity. (Arterioscler Thromb Vasc Biol. 2007;27:2184-2190.)

Key Words: UCHL1 ■ NF-κB ■ deubiquitinating enzyme ■ atherosclerosis

The vasculature is capable of sensing changes within its milieu, integrating these signals by intercellular communication, and changing itself through the local production of mediators that influence structure as well as function (eg, “vascular remodeling”). In the process of vascular remodeling, nuclear factor-κB (NF-κB) plays a pivotal role in the regulation of coordinated transactivation of genes involved in the expression of interleukins, intercellular adhesion molecules, vascular cell adhesion molecules, and endothelial leukocyte adhesion molecules. NF-κB activity is regulated in the cytoplasm through its association with IκB-α which is regulated by ubiquitin-proteasome system. Because phosphorylation of IκB-α is forwarded to be ubiquitinated by ubiquitinating enzymes and degraded by proteasomes, the ubiquitin-proteasome system is a critical mediator of NF-κB activity. Ubiquitin-mediated protein degradation plays a crucial role in various cellular processes, including signal transduction, cell differentiation, and stress response. By contrast, the deubiquitination system prevents protein degradation by reversing the ubiquitination process via the disassembly of the poly-ubiquitin chain, recycling active ubiquitin by the removal of ubiquitin from its covalently linked protein, and generating monomeric ubiquitin from its precursor fusion protein. Ubiquitinated IκB-α is also able to escape from degradation by deubiquitination. Regardless, regulation of transcription factor NF-κB remains the most important link between the ubiquitin-proteasome system and inflammation. Even though the proteasome inhibitor MG132 effectively reduces neointima formation, which corresponds to strong antiproliferative, antiinflammatory, and proapoptotic effects, it remains unclear whether deubiquitinating enzyme is involved in vascular remodeling.

We recently used a functional gene screening system with the HVJ-E vector to identify ubiquitin C-terminal hydrolase L1 (UCHL1) as an antiremodeling factor that is ex-
pressed in human endothelial cells. UCHL1 is a causative gene in Parkinson disease (PD), which is a neurodegenerative disorder associated with dysfunction of the ubiquitin-proteasome system.8 The goal of the present study was to clarify the role of the UCHL1 gene in vascular remodeling by evaluating nuclear factor-κB (NF-κB) inactivation in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs).

**Methods**

**Functional Screening Using Hemagglutinating Virus of Japan Envelope (HVJ-E)**

Several kinds of cells were maintained as previously described.9 Functional screening of a cDNA library using HVJ-E vector was performed as previously described10 using human umbilical venous endothelial cell (HUVEC) cDNA Library11 (a kind gift from Prof Hiroshi Nojima, Research Institute for Microbial Diseases, Osaka University, Japan).

**Plasmid DNA Construction**

Gateway cloning technology (Invitrogen) was used to construct expression vectors in accordance with the manufacturer’s instructions. Briefly, human UCHL1 and bovine eNOS were subcloned into D-TOPO to construct entry vectors and were then transferred into the mammalian expression vector, pcDNA3.1 or pCAGGS,12 through a LR-recombination reaction, creating pcDNA3.1-UCHL1, pCAGGS-FLAG-UCHL1, and pCAGGS-eNOS.

**Northern and Western Blotting and Immunoprecipitation**

Northern and Western blotting was performed as previously described.13,14 Cells were subjected to immunoprecipitation and immunoblot analysis as previously described.15 Of the 200 μL of cell lysates (50 μg), 10 μL was used for analysis of the relative expression levels of IκB-α and internal control proteins, and the remainder of the cell lysates was used for immunoprecipitation experiments with 0.5 mg anti-IκB-α (1:1000, sc-371, Santa Cruz Biotechnology Inc) and after immunoblotting with anti-ubiquitin (Ub) (1:2500, sc-8017, Santa Cruz Biotechnology Inc) and after immunoblotting with anti-ubiquitin (Ub) (1:2500, sc-8017, Santa Cruz Biotechnology Inc).

**Immunostaining**

Rat balloon-injured carotid artery was prepared as previously described.16 At least 3 individual sections (4 μm) from the middle of the injured arterial segments were fixed in 4% PFA for 15 minutes and then immunostained as previously described.17 All experimental protocols were approved by the Osaka University Graduate School of Medicine Standing Committee on Animals. The tissue specimens were obtained from symptomatic patients who underwent carotid endarterectomy for high-grade carotid stenosis. Written informed consent was obtained from all patients, and all protocols were approved by the Ethics Committee of Osaka University.

**In Vivo Gene Transfer Into Rat Balloon-Injured Carotid Artery**

The HVJ-liposome method was used for transfection of plasmid DNA in rat carotid artery as previously described.16,18 Briefly, a cannula was introduced into the common carotid artery through the external carotid artery. Then, 200 μL of HVJ-liposome complex was infused into the segment and incubated for 10 minutes at room temperature.

**Statistical Analysis**

All values are expressed as mean±SD. Data were compared using the ANOVA followed by Dunnett test for pair-wise comparisons against “control” and by Tukey test for multiple comparisons. All statistical analysis was performed using Stat-View 5.0 software.
and HASMCs (supplemental Figure IIB). Treatment of HAEcs and HASMCs with several growth factors or cytokines (ie, human recombinant epidermal growth factor [EGF: 10 ng/mL], fibroblast growth factor-2 [FGF2: 10 ng/mL], vascular endothelial growth factor [VEGF: 10 ng/mL], TNF-α [TFN-α: 10 ng/mL], and lipopolysaccharide [LPS: 30 ng/mL], angiotensin II [AngII: 1 μmol/L], hydrogen peroxide [H2O2; 100 μmol/L]) for 24 hours did not significantly alter UCHL1 mRNA levels (Figure 1A and supplemental Figure IIB). Treatment of cultured VSMCs with TNF-α (10 ng/mL) for 24 and 48 hours did not significantly alter UCHL1 mRNA levels, whereas long-term treatment (96 hours) with TNF-α (10 ng/mL) significantly increased UCHL1 mRNA levels (3.63±0.25-fold; P<0.01; Figure 1B).

Role of UCHL1 in Cultured Vascular Endothelial Cells

Treatment of BAECs or HAEcs with TNF-α (10 ng/mL) resulted in a significant increase in NF-κB activity, whereas overexpression of UCHL1 significantly attenuated both basal and TNF-α-induced NF-κB activity (56% inhibition in BAECs and 85% inhibition in HAEcs; P=0.05, compared with control under TNF-α treatment). In fact, the inhibitory effect of UCHL1 was similar to that of eNOS (Figure 2A and supplemental Figure IIIA). In HAEcs, immunofluorescent staining with anti-p65 antibody showed that p65, NF-κB subunit, was rapidly translocated into nucleus after the treatment of TNF-α for 30 minutes, whereas in UCHL1-transfected ECs, p65 remained to be located in the cytoplasm after the same treatment (supplemental Figure IVA). Although the treatment of TNF-α is known to decrease eNOS expression through NF-κB activation,21 overexpression of UCHL1 attenuated TNF-α-induced decreases in eNOS expression (supplemental Figure VA). Similarly, treatment of ECs with 2 different type of proteasome inhibitors, aspirin or MG132 attenuated TNF-α-induced eNOS downregulation (supplemental Figure VA).

As for the expression of NF-κB-driven gene, overexpression of UCHL1 in ECs attenuated TNF-α–induced expression of inducible NOS (iNOS; 54% inhibition), Manganese superoxide dismutase (Mn-SOD; 35% inhibition), heme oxygenase-1 (HO-1; 46% inhibition), vascular cell adhesion molecule-1 (VCAM-1; 74% inhibition), intercellular adhesion molecule-1 (ICAM-1; 57% inhibition), and E-selectin (37% inhibition), respectively (P<0.05 compared with control), as assessed by quantitative real-time–polymerase chain reaction (PCR; supplemental Figure VB).

We further examined how UCHL1 induced the NF-κB inactivation. IκB-α was phosphorylated and downregulated at 5 minutes after treatment with human recombinant TNF-α (10 ng/mL) in BAECs (data not shown). Five minutes after treatment with TNF-α, IκB-α expression was relatively high in UCHL1-overexpressed BAECs despite the fact that phospho-IκB-α expression was similar with or without overexpressed UCHL1 (Figure 2B and supplemental Figure IVB). Overexpression of UCHL1 resulted in decreased basal ubiquitination of IκB-α and higher protein levels of IκB-α (Figure 2C and supplemental Figure IVB).

Role of UCHL1 in Cultured Smooth Muscle Cells

Treatment of A7r5 (embryonic rat aortic smooth muscle cells) or HASMCs with TNF-α (10 ng/mL) for 12 hours significantly upregulated NF-κB activity, whereas overexpression of the UCHL1 gene significantly attenuated NF-κB activity, particularly in TNF-α–stimulated samples (46% inhibition in A7r5 and 63% inhibition in HASMCs; P<0.05, compared with control under TNF-α treatment) as shown in Figure 3A and supplemental Figure IIIIB. In fact, the inhibitory effect of UCHL1 was almost similar to that of eNOS.

Suppression of UCHL1 expression in A7r5 using siRNA was confirmed by real-time RT-PCR (73% inhibition, relative expression to GAPDH: control, 1.0±0.63; siRNA, 0.27±0.12). Indeed, the transfection of siRNA in A7r5 cells...
resulted in an increase in NF-κB activity regardless of TNF-α (10 ng/mL) stimulation for 12 hours (supplemental Figure VIA). In the gel shift mobility assay, overexpression of UCHL1 gene also attenuated the binding of NF-κB to DNA consensus sequence, and suppression of UCHL1 increased the binding of NF-κB (Figure 3B). They were consistent with the results obtained in experiments with the luciferase gene driven by the NF-κB binding site. Overexpressed UCHL1 attenuated TNF-α-induced cytokine expression (66% inhibition in TNF-α, 54% inhibition in interleukin [IL]-6, and 57% inhibition in matrix metalloprotease [MMP]-9; P<0.05 compared with control), as quantified by real-time PCR (supplemental Figure VIB).

Expression of UCHL1 in Intact and Injured Arteries
Northern blot analysis demonstrated mRNA expression of human UCHL1 in aorta (supplemental Figure VIIA). Immunostaining analysis showed that UCHL1 was expressed in whole brain, brain microvessels, rat common carotid artery, and rat abdominal artery (supplemental Figures VIIA, VIIIA, and VIIIIB).

UCHL1 expression was high in the neointimal smooth muscle cells of balloon-injured carotid artery, as confirmed by immunostaining with the VSMC specific marker, anti–α-SM actin (Figure 4A). The mRNA levels of UCHL1 were increased 25-fold compared with sham-operated common carotid artery quantified by real-time PCR (P<0.05). UCHL1 was also expressed in ECs and VSMCs in atherosclerotic lesions from human carotid arteries (Figure 4B).
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overexpressed UCHL1 attenuates NF-κB activation in vivo via stabilization of IkB-α, possibly through the deubiquitination of IkB-α. Thus, UCHL1 may reduce NF-κB-driven cytokines, such as inflammatory cytokines or adhesion molecules, and increase eNOS protein.

Figure 5. Effects of UCHL1 on balloon-injured arteries. A, Effect of overexpressed UCHL1 on neointimal formation in rat balloon injury model at 2 weeks after transfection. The panel shows the representative cross sections of balloon-injured vessels by hematoxylin-and-eosin staining (×40 magnification). "Control" indicates the transfection of LacZ plasmid. "UCHL1" indicates the transfection of UCHL1 plasmid. B, Effect of overexpressed UCHL1 on NF-κB inactivation in rat balloon injury model at 1 week after transfection, as assessed by immunostaining with anti-p50 (NF-κB subunits) antibodies recognizing the epitopes overlapping the nuclear location signal of the subunit of NF-κB heterodimer, and fairly selectively bind to the activated form of NF-κB. Thus, these results suggest that overexpressed UCHL1 attenuates NF-κB activation in vivo (Figure 5B and supplemental Figure X).

Functional Analysis of UCHL1 In Vivo
Expression of transfected UCHL1 gene was confirmed by RT-PCR and immunohistochemistry in carotid arteries 3 days after balloon injury (data not shown). As shown in Figure 5A, overexpression of UCHL1 plasmid resulted in significant inhibition of neointimal formation, as quantified by measurement of neointimal and medial area and by calculation of the ratio of neointimal-to-medial area at 2 weeks after balloon injury (supplemental Figure IX, P<0.01). Accompanied with an inhibition of neointimal formation by transfection of UCHL1 plasmid DNA, the inhibition of NF-κB activation was examined assessed by immunostaining at 1 week after transfection. These anti-p50 and anti-p65 (NF-κB subunits) antibodies recognize the epitopes overlapping the nuclear location signal of the subunit of NF-κB heterodimer, and fairly selectively bind to the activated form of NF-κB. Thus, these results suggest that overexpressed UCHL1 attenuates NF-κB activation in vivo (Figure 5B and supplemental Figure X).

Discussion
The present study revealed that UCHL1 was expressed in atherosclerotic lesion and that UCHL1 may participate in vascular remodeling via inhibition of NF-κB activity. Specifically, UCHL1 attenuated TNF-α–induced NF-κB activity and increased eNOS expression, which may directly attenuate atherosclerosis leading to the reduction of ischemic vascular disease.21 Indeed, overexpressed UCHL1 reduced neointima formation in balloon-injured artery. The present study provides the first evidence that a deubiquitinating enzyme can modulate vascular remodeling.

UCHL1 (also known as pGp9.5) is predominantly expressed in central and peripheral neurons. Mutations in the UCHL1 gene have been identified in a single German PD family with a reduced penetrance inheritance pattern.24 Furthermore, a polymorphism in exon 3 of the UCHL1 gene (G18 years) is associated with a reduced susceptibility to PD in some populations25,26 and may be related to onset of Huntington’s disease.27 UCHL1 hydrolyzes small C-terminal adducts of ubiquitin to generate the ubiquitin monomer, making it an important component of the ubiquitin-proteasome system. Further, the ubiquitin-proteasome path-
way has an important role in vascular remodeling\(^8\) and cardiac fibrosis,\(^28\) as demonstrated by the observation that treatment of cardiovascular cells with proteasome inhibitors results in strong antiproliferative, antiinflammatory, and pro-apoptotic effects.\(^6\) In this study, although overexpressed UCHL1 attenuated the TNF-\(\alpha\)-induced inflammatory action, endogenous UCHL1 may not affect the first action of TNF-\(\alpha\)-induced inflammation because the induction of UCHL1 expression by TNF-\(\alpha\) was delayed. This suggests that UCHL1 cannot be directly targeted, but is chronically upregulated by several stimulants in vascular remodeling. This is consistent with recent studies that have demonstrated that full-length UCHL1 is a major target of oxidative damage and that UCHL1 is downregulated in the brain of patients with Alzheimer or Parkinson disease.\(^29\) Thus, deubiquitinating enzymes, such as UCHL1, may act as suppressors during the activation of ubiquitin-proteasome system in atherosclerosis lesions.

Recent evidence has shown that UCHL1 is also highly expressed in carcinomas of various tissue origins, including those from brain, lung, breast, kidney, colon, prostate, pancreas, and mesenchymal tissues.\(^30\) Indeed, in 1 lung cancer cell line, UCHL1 interacts with the Jun activation domain binding protein, JAB1, and a cyclin dependent kinase inhibitor, p27 (Kip1).\(^31\) Further, UCHL1 exerts an antiproliferative response, and its expression may be induced as a response to tumor growth.\(^32\) This is consistent with observations from the present study that overexpressed UCHL1 attenuated neointimal formation in balloon-injured artery. Interestingly, aspirin and statins, 2 of the most successful drugs in the attenuation of cardiovascular events, both exert an inhibitory effect on the proteosome.\(^22,33\) From the perspective of the ubiquitin-proteasome system, the pleiotropic effect of aspirin was reported in the upregulation of eNOS expression.\(^22\) In combination with the observation that aspirin upregulated Lb-\(\alpha\) protein through inhibition of ubiquitin-proteasome system,\(^22\) the present study may provide a new understanding of the mechanisms by which pharmacological agents may prevent atherosclerosis. Further study to determine the mechanisms by which UCHL1 or another deubiquitinating enzyme contributes to the process of cardiovascular diseases would be of benefit.

In conclusion, this study demonstrated expression of UCHL1 in vascular endothelial cells and smooth muscle cells and the antiinflammatory action of UCHL1 in the process of vascular remodeling. The UCHL1 gene may represent a novel therapeutic target for the attenuation of atherosclerosis and the prevention of cardiovascular events.

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**Disclosures**

None.

**References**


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Supplement

Methods

Materials

Recombinant fibroblast growth factor-2 (FGF2), tumor necrosis factor (TNF)-α, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) were obtained from PeproTec (London, UK). Other material was obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

HAEC (human aortic endothelial cells), HASMC (human aortic smooth muscle cells), and HUVEC (human umbilical venous endothelial cell) were purchased from Clonetics Corp. (Palo Alto, CA) and were maintained as previously described 1. BAEC (bovine aortic endothelial cells) and A7r5 (embryonic thoracic aorta, smooth muscle, DB1X rat) were purchased from Clonetics Corp. and the American Type Culture Collection (ATCC), respectively, and maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂ with medium changes every two days.

Functional screening using Hemagglutinating Virus of Japan Envelope (HVJ-E)

Briefly, HUVEC cDNA Library (a kind gift from Prof. Hiroshi Nojima, Research Institute for Microbial Diseases, Osaka University) was amplified once using
the Plasmid Giga Kit (QIAGEN, Valencia, CA), and infused into HVJ-E vectors. A7r5 (10³ cells/well) seeded on 96-well plates were transfected with cDNA library infused-HVJ-E and then treated with human recombinant platelet derived growth factor (50 ng/mL) for 24 hours. Candidate genes were obtained from the lowest proliferative cells evaluated by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI), followed by isolation of transfected genes by transformation into E. coli.

Isolated candidate genes were further evaluated by assessment of NF-κB activity. In BAEC or A7r5, each candidate gene was co-transfected with the luciferase gene driven by the NF-κB binding site (BD Bioscience Clontech, Palo Alto, CA) and PRT-TK plasmids (Promega, Madison, WI) using lipofectAMINE 2000 (Invitrogen, Grand Island, NY). After transfection, luciferase activity was measured with the Dual-Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s instructions following treatment for 24 hours in serum-free DMEM. NF-κB activity was measured with the treatment of human recombinant TNF-α (10 ng/ml).

**Northern blotting**

Total RNAs were prepared from HAEC and HASMC with RNeasy Mini Kit (Quiagen) under various conditions. Equal aliquots of total RNA (5 µg) were separated by 1% formaldehyde-agarose gel electrophoresis, and hybridization and washing were performed. Full length of UCHL1 cDNA was used as a probe. Loading conditions were determined by reprobing with GAPDH or β-actin.
MTN™ Blot (#7791-1, Clontech Laboratories, Inc.) was prepared from high-quality poly(A)+ RNA and normalized against a β-actin or GAPDH hybridization signal (Clontech) according to the manufacturer’s instructions in order to investigate gene expression of UCHL1 in human cardiovascular systems, including the aorta.

**Western blotting**

Briefly, cell extracts were prepared with lysis buffer (RIPA buffer: 150 mM NaCl, 1% NP-40, 0.5% deoxycholate-Na, 0.1% SDS, 50 mmol/l Tris-HCl (pH 8.0), and EDTA-free protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany)). Samples containing 5-10 µg protein were separated on 10% sodium dodecylsulfate polyacrylamide (SDS/PAGE) gels and transferred to nitrocellulose membranes (Hybond ECL™; Amersham Life Science Inc., Arlington Heights, Illinois), and incubated with a monoclonal antibody to eNOS (1:1000, BD Transduction Laboratories), UCHL1 (1:2000, Sigma), a polyclonal antibody to phosphospecific or total inhibitory kappa B (IκB)-α (1:1000, Cell Signaling TECHNOLOGY, Beverly, MA), and β-actin (1:5000, Sigma) at 4°C overnight. The membranes were then washed and incubated with a 1:2000 dilution of mouse or rabbit IgG horseradish peroxidase-conjugated antibody (Amersham). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham) with Hyperfilm™-MP (Amersham).

**Gel Mobility Shift Assay**

The nuclear extract was prepared from cultured A7r5 and BAECs with over-expressed or knocked-down UCHL1 using NE-PER™ (Promega) according to the manufacturer’s instructions. Gel mobility shift assay was performed using a Gel Shift Assay System (Promega) according to the manufacturer’s instructions. Briefly,
NF-κB-consensus oligodeoxynucleotide (5'-AGTTGAGGGGACTTTCCAGGC-3') was labeled with $^{32}$P and purified using a Nick column (Pharmacia Biotech). Binding reactions (10 µl), including $^{32}$P-labeled probe (0.5 to 1 ng; 10,000 to 15,000 cpm), were incubated with 5 µg of nuclear extract in 1 mmol/l MgCl$_2$, 50 mmol/l NaCl, 50 mmol/l Tris-HCl (pH 7.5), 0.5 mmol/l EDTA, 0.5 mmol/l DTT, 4% glycerol, 0.05 mg/ml poly(deoxyinosinic-deoxycytidylic) acid in a total of 10 µl for 30 min at room temperature, and then loaded onto a 4% polyacrylamide gel. Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabelled consensus oligonucleotides. The gels were subjected to electrophoresis, drying, and autoradiography.

**Real time reverse transcription-polymerase chain reaction (RT-PCR)**

Human or rat UCHL1 expression was measured by real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA of cells or tissue samples was extracted using RNeasy Mini Kit (Qiagen) or the ISOGEN (NIPPON GENE, Toyama, Japan). Complementary DNA was synthesized using the Thermo Script RT-PCR System (Invitrogen, Carlsbad, CA). Relative gene copy numbers of UCHL1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time RT-PCR using TaqMan Gene Expression Assays (human UCHL1: Hs00188233, rat UCHL1: Rn00568258, human GAPDH: Hs99999905, rat GAPDH: Rn99999916, rat TNF-α: Rn99999017, rat interleukin-6: Rn00561420, rat matrix metalloproteinase-9: Rn00579162, 18S rRNA: Hs99999901, Applied Biosystems, Foster City, CA) or Cyber Green Gene Expression Assays using SYBR Premix Ex Taq™ (TAKARA BIO INC.). The absolute number of gene copies was normalized using GAPDH and standardized by a sample standard curve. The information of
specific primers for Cyber Green Gene Expression Assay is as follows:

Bovine GAPDH  
- Forward: 5’-GAGGGACTTATGACCACCTGTCAC-3’
- Reverse: 5’-GGGCCATCACCAGTTCTCTCG-3’

Bovine ICAM-1  
- Forward: 5’-TCACCCTATCTGGTCCCGAG-3’
- Reverse: 5’-GAGGTTCTCCACCACAGGCCTGC-3’

Bovine VCAM-1  
- Forward: 5’-AATTAACTTGTTCAAGAGAAAAACTTTACTGTT-3’
- Reverse: 5’-CACGACTGAGTCCACCAACC-3’

Bovine E-selectin  
- Forward: 5’-CTACTGCTGGAGTCTCCCTTGTGAC-3’
- Reverse: 5’-GGCTTGAGCAGCTGCTGGCAGGAGA-3’

Bovine iNOS  
- Forward: 5’-CTGGAGGAAGTGGGCAGAAG-3’
- Reverse: 5’-TCTGCTTCTGGAAACTGTGGAG-3’

Bovine MnSOD  
- Forward: 5’-CGCTGGAGAAGGTTGATGT-3’
- Reverse: 5’-CCACCGTTAGGGCTCAGATT-3’

Bovine HO-1  
- Forward: 5’-CCAGCTGGTTTGAGGAGGATT-3’
- Reverse: 5’-CCAAGTCTTTGCACTTTGCTG-3’

RNA Interference and oligodeoxynucleotides

The siRNA for rat UCHL1 or scramble siRNA were designed using the siSNIPER system (Genomidea, Inc, Osaka and Mitsubishi Space Software Co., Ltd., Japan). The sequence of rat UCHL1 (sense) was 5’-UGGCCAAUAACCAAGACAATT-3’ and the sequence of scrambled siRNA (sense) was 5’-UUCCUCAAAGAUGCGUGUTT-3’. The siRNA oligonucleotides were transfected into A7r5 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.
**Immunohistochemical staining and rat carotid balloon injury**

Cells on glass coverslips were fixed in 4% paraformaldehyde (PFA) for 15 minutes and then permeabilized with 0.2% Triton X-100 for 5 minutes. After blocking in 5% skim milk, samples were incubated with anti-UCHL1 antibody (1:500, Sigma), anti-p65 antibody (1:100, sc-372, Santa Cruz Biotechnology Inc.), or monoclonal anti-FLAG M2 antibody (1:500 Sigma) at 4°C overnight. The sequence of immunogen used for this anti-UCHL1 antibody has no homology with other known UCH-L isoforms. Corresponding secondary antibodies were labeled with AlexaFluor 488 or 546 (1:200, Molecular Probes, Eugene, OR).

Wild-type male Sprague-Dawley rats (weight, 400 to 500 g; Charles River Breeding Laboratories) were anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally. Rat balloon-injured carotid artery was prepared as previously described. Briefly, a 2F Fogarty catheter was introduced into the common carotid artery through the external carotid artery, and vascular injury of the common carotid artery was induced by the passage and inflation of a balloon catheter three times. Morphological study of the injured arteries was performed 1 and 2 weeks after injury. At least three individual sections (4 µm) from the middle of the injured arterial segments were fixed in 4% PFA for 15 minutes and then immunostained as previously described. Sections were stained with anti-smooth muscle (SM) actin antibody (1: 200, Sigma) and anti-UCHL1 antibody (1: 100, Sigma), and further incubated with corresponding secondary antibodies labeled with AlexaFluor 488 or 546, respectively (1:200) and DAPI. One week after gene transfection, sections were stained with anti-p50 (1:100, sc-114 Santa Cruz Biotechnology Inc), anti-p65 (1:100, CHEMICON International, Inc.),
anti-ICAM-1 (1:100, sc-1511, Santa Cruz Biotechnology Inc), and anti-MMP9 (1:200, Abcam Inc.) antibodies. Anti-p50 and anti-p65 antibodies recognize epitopes overlapping the nuclear location signal of the subunit of NF-κB heterodimer. Thus, these antibodies selectively bind to the activated form of NF-κB. To rule out non-specific staining in each immunofluorescence experiment, a negative control was performed without the addition of primary antibody, and all negative controls in this study were not stained (data not shown). All experimental protocols were approved by the Osaka University Graduate School of Medicine Standing Committee on Animals.

For immunohistological studies of human atherosclerotic plaques, tissue specimens were obtained from symptomatic patients who underwent carotid endarterectomy for high-grade carotid stenosis. After surgery, plaques were fixed in formalin and embedded in paraffin for histology. Sections were serially cut at 5 µm, mounted on lysine-coated slides, and immunofluorescent staining was performed as aforementioned, after deparaffinizing, hydrating, and treating for antigen retrieval using HistoVT One (Nacalai Tesque, Kyoto, Japan). Written informed consent was obtained from all patients, and all protocols were approved by the Ethics Committee of Osaka University.

**In vivo gene transfer into rat balloon-injured carotid artery**

The HVJ-liposome method was used for transfection of plasmid DNA in rat carotid artery. A cannula was introduced into the common carotid artery through the external carotid artery. Then, 200 µL of HVJ-liposome complex was infused into the segment and incubated for 10 minutes at room temperature. After 10-minute incubation, the infusion cannula was removed. After transfection, blood flow to the common carotid
artery was restored by release of the ligatures, and the wound was closed. Each carotid artery was processed for immunochemical staining at 7 days after injury and transfection and for morphological study at 2 weeks after injury. The cross sections of carotid artery were stained with hematoxylin and eosin and photographed. The intimal and medial cross-sectional area of the carotid arteries was measured using Image J (Bethesda, MD), and the intima/media ratios of cross sections were calculated. At least three individual sections from the middle of the transfected arterial segments were analyzed.

References
**Figure Legend**

**Supplement Figure 1.**  NF-κB activity evaluated by the luciferase gene driven by the NF-κB binding site in (A) HUVEC, (B) HASMC, (C) BAEC and (D) A7r5 in growth medium for 24 hours.

“control” indicates over-expressed GFP gene, “UCHL1” indicates over-expressed human UCHL1 gene. *p<0.05 vs. control. N=4 per group in triplicate.

(E) Representative EMSA for NF-κB activity in BAEC. “competitor” indicates addition of non-RI labeled consensus oligodeoxynucleotide of NF-κB binding sequence in “control” sample. “FP” indicates free probe (non-binding RI labeled oligodeoxynucleotide). Experiments were performed in triplicate.

**Supplement Figure 2.**  Expression of UCHL1 in vascular cells

A) Immunostaining analysis of UCHL1 in HAEC and HASMC cultured in 5% FBS. “DAPI” indicates nuclear staining (blue), “UCHL1” indicates staining with anti-UCHL1 antibody, and “Merge” indicates staining with both. Bar (white) = 10 µm.

B) Representative northern blot of human UCHL1 in HASMC. Corresponding β-actin or GAPDH expression was used to standardize loading. “control” indicates no stimulation. Cultured cells were treated with human recombinant TNF-α (10 ng/ml), LPS (30 ng/ml), angiotensin II (1 µmol/l), or hydrogen peroxide (H2O2; 100 µmol/l) for 24 hours. Experiments were performed in duplicate.

**Supplement Figure 3.**

(A) and (B) Effect of over-expressed human UCHL1 on NF-κB activity, as evaluated by the luciferase gene driven by the NF-κB binding site in (A) HAEC and (B) HASMC.
“Control” indicates over-expressed GFP gene, “UCHL1” indicated over-expressed human UCHL1 gene, and “eNOS” indicates over-expressed eNOS gene. “TNF-α” indicates treatment with human recombinant TNF-α (10 ng/ml) for 12 hours. *P<0.05 vs. control. #P<0.05 vs. TNF-α (-). N=6 per group in triplicate.

**Supplement Figure 4.**

A) Immunostaining analysis of NF-κB subunit, p65, in HAEC with treatment of TNF-α (10 ng/ml) for 30 minutes (x 900 magnification). Upper panels show immunofluorescent staining in GFP-tranfected cells (control) and lower panels in FLAG-tagged-UCHL1-transfected cells (UCHL1). “DAPI” indicates nuclear staining (blue), “p65” indicates staining with anti-p65 antibody (red), and “Merge” indicates staining with both. Inserts in merge panels show GFP (green) in control and immunofluorescent staining with anti-FLAG antibody (green) in UCHL1. “TNF-α” indicates treatment of TNF-α for 30 minutes.

(B) Left panel shows the quantification by measured density of IκB-α protein and in western blotting. IκB-α protein levels were corrected using β-actin as an internal control; AU, arbitrary unit; N=5 per group, *P<0.05 vs. control. Right panel shows the quantification by measured density of ubiquitinated IκB-α protein in western blotting. Ubiquitinated IκB-α protein levels were corrected using GAPDH as an internal control; AU. Arbitrary unit, N=5 per group, *P<0.05 vs. control.

**Supplement Figure 5.**

A) Representative western blot of eNOS protein. “control” indicates over-expressed GFP gene, “UCHL1” indicates over-expressed human UCHL1 gene, “aspirin” indicates
treatment with aspirin (10 mmol/l), and “MG132” indicates treatment with MG132 (10 µmol/l) with or without co-treatment with human recombinant TNF-α (10 ng/ml) for 24 hours. Corresponding β-actin expression was used to standardize loading. Experiments were performed in triplicate.

B) Fold increase of iNOS (inducible nitric oxide synthase), Mn-SOD (Manganese Superoxide Dismutase), HO-1 (heme oxygenase-1), VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1) and E-selectin expression induced by TNF-α treatment (10 ng/ml) for 6 hours in GFP or human UCHL1 gene-over-expressed BAEC evaluated by quantitative real time RT-PCR. “control” indicates over-expressed GFP gene, “UCHL1” indicates over-expressed human UCHL1 gene. N=6 per group in duplicate. *P<0.05 vs. control.

Supplement Figure 6.
A) Effect of suppressed UCHL1 expression by siRNA on NF-κB activity evaluated by the luciferase gene driven by the NF-κB binding site in A7r5. “control” indicates transfection of scramble siRNA, “siRNA” indicated suppressed UCHL1 expression by transient transfection of siRNA for UCHL1. “TNF-α” indicates treatment with human recombinant TNF-α (10 ng/ml) for 12 hours. *P<0.05 vs. control. #P<0.05 vs. TNF-α (-). N=6 per group in triplicate.

B) Fold increase of TNF-α, IL-6, and MMP-9 expression induced by TNF-α treatment (10 ng/ml) for 6 hours in control gene- or human UCHL1 gene-over-expressed A7r5 evaluated by quantitative real time RT-PCR. “Control” indicates over-expressed GFP gene, “UCHL1” indicates over-expressed human UCHL1 gene. N=4 per group in triplicate. *P<0.05 vs. control.
Supplement Figure 7.

A) Representative northern blot of human UCHL1 in fetal heart, adult heart and aorta. Corresponding GAPDH expression was used to standardize loading. Experiments were performed in duplicate.

B) Representative pictures of immunofluorescent staining in rat common carotid artery (x100 magnification). “DAPI” indicates nuclear staining (blue), “α-SM actin” indicates the staining with anti-α-smooth muscle (SM) actin antibody (green), “UCHL1” indicates the staining with anti-UCHL1 antibody (red), and “Merge” indicates staining with all three.

Supplement Figure 8.

A) Representative pictures of immunofluorescent staining with anti-UCHL1 antibody in mouse brain and a micro-vessel. Upper panel shows immunofluorescent staining (x100 magnification) in the cortex of mice brain, and lower panel shows immunofluorescent staining of the micro-vessel of mouse brain (x200 magnification). “DAPI” indicates nuclear staining (blue), “UCHL1” indicates the staining with anti-UCHL1 antibody, and “Merge” indicates staining with both.

B) Representative pictures of immunofluorescent staining with anti-UCHL1 antibody in rat abdominal aorta (x100 magnification). “DAPI” indicates nuclear staining (blue), “α-SM actin” indicates the staining with anti-α-SM actin antibody, “UCHL1” indicates the staining with anti-UCHL1 antibody, and “Merge” indicates staining with all three.

Supplement Figure 9.
The panels show the quantification of intimal area, medial area, and ratio of neointimal-to-medial area in rat balloon injury model at 2 weeks after transfection. “Control” indicates the transfection of LacZ plasmid. “UCHL1” indicates the transfection of UCHL1 plasmid. *P<0.01 vs. control. “NS” indicates not significant. Control group contains 12 animals; UCHL1 group contains 14 animals.

**Supplement Figure 8.**

Representative pictures of immunostaining with anti-p50 and anti-MMP-9 antibody to evaluate the effect of over-expressed UCHL1 on NF-κB inactivation in rat balloon injury model at 1 week after transfection. Upper panel shows representative cross sections stained with anti-p50 antibody (red: x 200 magnification) and lower panel stained with anti-MMP9 antibody (red: x200 magnification). “Control” indicates the transfection of LacZ plasmid. “UCHL1” indicates the transfection of UCHL1 plasmid. “DAPI” indicates nuclear staining (blue). “Merge” indicates staining with both. Arrows (white) indicates neointimal formation.
Supplement Figure 1

(A) and (D) show bar graphs comparing the fold increase of control and UCHL1 treatments. (A) demonstrates a significant increase (*) in the UCHL1 group compared to control. (D) follows the same pattern.

(B) also presents similar findings, with a significant increase (*) in the UCHL1 group compared to control.

(C) further supports this pattern, showing a significant increase (*) in the UCHL1 group.

(E) includes a gel image with lanes for control, UCHL1, and competitor, labeled with FP.
Supplement Figure 2

(A)

HAEC

DAPI  UCHL1  Merge

HASMC

(B)

UCHL1

GAPDH

control  LPS  TNF-α  AngII  H₂O₂
Supplement Figure 3

(A) Fold increase in UCHL1 and eNOS expression in control and TNF-α-treated cells.

(B) Fold increase in UCHL1 and eNOS expression in control and TNF-α-treated cells.
Supplement Figure 4

(A) DAPI | p65 | Merge

control

UCHL1

(B)

\[ \text{normalized AU (fold)} \]

\[ \text{I}_\text{κB-α} \]

\[ \text{ubiquitinated I}_\text{κB-α} \]
Supplement Figure 5

(A) Control UCHL1

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<thead>
<tr>
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<th>UCHL1</th>
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<tr>
<td>TNF-α</td>
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<td>+</td>
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<td>eNOS</td>
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<tr>
<td>β-actin</td>
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Aspirin (10 mmol/l) MG132 (10 µmol/l)

<table>
<thead>
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(B)

- iNOS
- Mn-SOD
- HO-1
- VCAM-1
- ICAM-1
- E-selectin

Fold increase of TNF-α-induced gene expression

Control UCHL1

* indicates significant difference
Supplement Figure 6

(A)

![Bar chart showing fold increase of TNF-α-induced gene expression with and without siRNA treatment.](image)

TNF-α (-)  TNF-α (+)

control  siRNA  control  siRNA

(B)

![Bar chart showing fold increase of TNF-α-induced gene expression for TNF-α, IL-6, and MMP-9 with and without UCHL1 knockdown.](image)
Supplement Figure 7

(A)

UCHL1

GAPDH

fetal heart  adult heart  aorta

(B)

DAPI

α-SM actin

UCHL1

Merge
Supplement Figure 8

(A) DAPI  UCHL1  Merge

(B) DAPI  α-SM actin
          UCHL1  Merge
Supplement Figure 9

Graphs showing the comparison of intimal area, medial area, and intima/media ratio between control and UCHL1 groups. The graphs indicate a significant difference (*p < 0.05) in intimal area and intima/media ratio, with no significant difference (NS) in medial area.
Supplement Figure 10

DAPI  active p50  Merge

control

UCHL1

DAPI  MMP-9  Merge

control

UCHL1