and oxidative stress contribute to tissue damage in several situations, such as ischemia–reperfusion injury in the brain, heart and kidney, future studies of CyPA-mediated function in appropriate models may reveal its significant role in other diseases. By blocking the vicious cycle that augments ROS production through the CyPA autocrine/paracrine signaling pathway, we may have a novel therapeutic tool for controlling cardiovascular diseases in the near future.

Acknowledgments

This work was supported by NIH grant HL49192 (B.C. Berk), AstraZeneca Research Grant (K. Satoh), grants-in-aid for scientific research from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan (K. Satoh), and Grants-in-aid for scientific research from the Ministry of Health, Labour, and Welfare, Tokyo, Japan. We are grateful to members of the Berk lab in the Aab Cardiovascular Research Institute at the University of Rochester School of Medicine for useful suggestions, especially the work performed by Duan-Fang Liao, Zheng-Gen Jin, Jun Suzuki, Tetsuya Matoba, and Patrizia Nigro.

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Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Cyclophilin A Mediates Vascular Remodeling by Promoting Inflammation and Vascular Smooth Muscle Cell Proliferation

Kimio Satoh, Tetsuya Matoba, Jun Suzuki, Michael R. O'Dell, Patrizia Nigro, Zhaoqiang Cui, Amy Mohan, Shi Pan, Lingli Li, Zheng-Gen Jin, Chen Yan, Jun-ichi Abe and Bradford C. Berk

Circulation. 2008;117:3088-3098; originally published online June 9, 2008;
doi: 10.1161/CIRCULATIONAHA.107.756106
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539

Cyclophilin A Mediates Vascular Remodeling by Promoting Inflammation and Vascular Smooth Muscle Cell Proliferation

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Background—Oxidative stress, generated by excessive reactive oxygen species, promotes cardiovascular disease. Cyclophilin A (CyPA) is a 20-kDa chaperone protein secreted from vascular smooth muscle cells (VSMCs) in response to reactive oxygen species that stimulates VSMC proliferation and inflammatory cell migration in vitro; however, the role CyPA plays in vascular function in vivo remains unknown.

Methods and Results—We tested the hypothesis that CyPA contributes to vascular remodeling by analyzing the response to complete carotid ligation in CyPA knockout mice, wild-type mice, and mice that overexpress CyPA in VSMC (VSMC-Tg). After carotid ligation, CyPA expression in vessels of wild-type mice increased dramatically and was significantly greater in VSMC-Tg mice. Reactive oxygen species–induced secretion of CyPA from mouse VSMCs correlated significantly with intracellular CyPA expression. Intimal and medial hyperplasia correlated significantly with CyPA expression after 2 weeks of carotid ligation, with marked decreases in CyPA knockout mice and increases in VSMC-Tg mice. Inflammatory cell migration into the intima was significantly reduced in CyPA knockout mice and increased in VSMC-Tg mice. Additionally, VSMC proliferation assessed by $Ki67^+$ cells was significantly less in CyPA knockout mice and was increased in VSMC-Tg mice. The importance of CyPA for intimal and medial thickening was shown by strong correlations between CyPA expression and the number of both inflammatory cells and proliferating VSMCs in vivo and in vitro.

Conclusions—In response to low flow, CyPA plays a crucial role in VSMC migration and proliferation, as well as inflammatory cell accumulation, thereby regulating flow-mediated vascular remodeling and intima formation. (***Circulation*. 2008;117:3088-3098.**)

Key Words: reactive oxygen species ■ vasculature ■ remodeling ■ atherosclerosis ■ restenosis

The interaction between endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) plays an important role in regulating vascular integrity. ECs secrete several vasoactive substances, including nitric oxide and prostacyclin, which maintain vascular integrity and limit intima formation.[1] VSMCs contain numerous sources of reactive oxygen species (ROS; ie, H_2O_2, O_2^-, and · OH), including NADPH oxidases, xanthine oxidase, the mitochondrial respiratory chain, lipoxygenases, and nitric oxide synthases.[2] It has become clear that increases in ROS represent 1 of the pathogenic mechanisms for vascular disease.[3,4] ROS have been implicated in the pathogenesis of intima formation in part by promoting VSMC growth,[5,6] as well as by stimulating proinflammatory events.[7–9] Recently, we proposed a pathogenic role for a newly discovered class of ROS mediators that we term SOXF, for *s*ecreted *ox*idative stress–induced *f*actors.[10,11] Among these factors, cyclophilin A (CyPA) expression is induced by ROS, and CyPA is secreted in response to ROS.[10–12] We demonstrated that CyPA stimulates proinflammatory signals in ECs and VSMCs, including expression of E-selectin and vascular cell adhesion molecule (VCAM)-1.[13] Furthermore, we showed that secreted CyPA stimulates the ERK1/2 (extracellular signal-regulated kinases) and JAK/STAT (Janus kinases/signal transducers and activators of transcription) pathways in vitro, thereby increasing DNA synthesis in VSMCs.[10] In addition to effects on vascular cells, CyPA has been shown to be a chemoattractant for inflammatory cells[14,15] and promotes activation of matrix metalloproteinases (MMPs), especially MMP-1 and MMP-9.[14,16] Therefore, CyPA is a key mediator that affects ECs, VSMCs, and

Received November 27, 2007; accepted March 24, 2008.

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The online-only Data Supplement, which contains a supplemental Methods section and Figures I through VI, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.756106/DC1.

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Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.107.756106**

inflammatory cell function during oxidative stress. Here, we tested the hypothesis that CyPA contributes to vascular remodeling by analyzing the response to complete carotid ligation in CyPA knockout ($CyPA^{-/-}$) mice, wild-type (WT) mice, and mice that overexpress CyPA specifically in VSMC (VSMC-Tg).

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Methods

CyPA Knockout Mice

All animal experiments were conducted in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Rochester (2002-135A). $CyPA^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Me) and were backcrossed to C57BL/6J mice for 7 generations. WT littermates ($CyPA^{+/+}$) were used as controls, and all mice were genotyped by polymerase chain reaction on tail-clip samples.

Generation of CyPA-Overexpressing Transgenic Mouse

We used a Cre/LoxP strategy to prepare CyPA transgenic mice. In brief, a $LacZ^{flox}$-CyPA construct was prepared with the pZ/EG vector (Data Supplement Figure I). The pZ/EG double-reporter construct was a kind gift from the Nagy laboratory.[17] This vector contains LacZ floxed by 2 loxP sites, driven by the chicken β-actin promoter and a cytomegalovirus enhancer with enhanced green fluorescent protein downstream.[18] We replaced enhanced green fluorescent protein with full-length WT mouse CyPA carrying a Flag tag to make the $LacZ^{flox}$-Flag-CyPA construct. Embryonic stem cells transfected by electroporation with linearized $LacZ^{flox}$-Flag-CyPA cDNA were screened by neomycin resistance and LacZ expression. Embryonic stem clones with a single copy by Southern blotting were used to generate chimeric mice by embryonic stem cell–embryo aggregation. The chimeric mice were bred to C57BL/6J mice to produce hemizygous transgenic offspring. Hemizygous offspring with germline transmission were identified by polymerase chain reaction of DNA harvested from tail snippets of weaned offspring. We obtained 9 germline mice from the 2A3 embryonic stem cell clone and 8 from the 3H9 embryonic stem cell clone. Transgenic mice were backcrossed to C57BL/6J mice for 7 generations to establish experimental lines.

VSMC-Specific Overexpression of CyPA

For VSMC-specific overexpression of CyPA in transgenic mice, the $LacZ^{flox}$-CyPA transgenic mouse and SM22α-Cre mouse (C57BL/6J background)[19] were crossed. Previously, we showed that expression of SM22α promoter when linked to LacZ was restricted to VSMCs in the day 12.5 embryo, without expression in other smooth muscle. Breeding of the $LacZ^{flox}$-CyPA mice to SM22α-Cre mice[19] resulted in excision of LacZ and expression of CyPA in VSMCs.

Complete Common Carotid Artery Ligation

Six- to 8-week-old male mice underwent complete carotid artery ligation.[20,21] Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The left common carotid artery was exposed through a small midline incision in the neck and was completely ligated with 6-0 silk just proximal to the carotid bifurcation (Data Supplement Figure IIA).[20,21] In the right common carotid artery (sham), the suture was passed under the exposed carotid artery but not tightened. Four groups of operated animals were processed for morphological studies at 14 days after the operation. Survival rate of the operated mice was >95%, and none of the mice showed any neurological deficits.

Morphometric Analysis

For morphological analysis, 48 animals were perfused with normal saline and fixed with 10% phosphate-buffered formalin at physiological pressure for 5 minutes.[22] The carotid arteries were harvested, fixed for 24 hours, and embedded in paraffin, and cross sections (5 μm) were prepared.[22] Because lesion thickness varies longitudinally, the entire length of the left and right carotid arteries was sectioned, and 5 sections located at 250-μm intervals from the carotid bifurcation were examined (Data Supplement Figure IIA). Vessel areas were measured with ImagePro Plus software (Media Cybernetics Inc, Silver Spring, Md) and morphological parameters calculated as described previously.[20,21] In brief, the intimal area was calculated as the internal elastic lamina area minus luminal area, the medial area was the external elastic lamina area minus the internal elastic lamina area, and the adventitial area was the vascular area minus the external elastic lamina area (Data Supplement Figure IIB).

Harvesting of Mouse Aortic Smooth Muscle Cells

Mouse aortic smooth muscle cells (MASMs) were isolated from each strain of mice (WT, $CyPA^{-/-}$, VSMC-Tg, and control mice) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37°C in a humidified atmosphere of 5% CO_2/95% air as described previously.[23,24] Passages 4 to 6 of MASMs at 70% to 80% confluence were used for experiments.

Preparation of Conditioned Medium

MASMs from each mouse strain were serum starved in DMEM for 24 hours and stimulated with 1 μmol/L LY83583 to generate intracellular ROS,[10,11] and medium was collected and centrifuged for 10 minutes at 800g to remove cell debris. The medium was concentrated 100-fold with a Centricon Plus-20 filter (Millipore Corp, Bedford, Mass) to yield concentrated conditioned medium (CM).[10,11]

Proliferation and Scratch-Wound Assays

MASMs were seeded in 96-well plates in DMEM supplemented with 10% FBS. For proliferation assays, medium was changed to DMEM without FBS and starved for 24 hours, then stimulated with CM for up to 5 days. CM was changed at day 3, and cells were counted at day 2 and day 5. For scratch wound, confluent cells were scratched with a pipette tip, and medium was replaced with CM from different MASMs (Tg, WT, and knockout [KO]), and cells were allowed to migrate for 24 hours. To block protein synthesis (cell proliferation), MASMs were pretreated with anisomycin (10 μmol/L) for 2 hours. MASMs were positive for smooth muscle actin (α-SMA; green).

Boyden Chamber Migration Assays

For the Boyden chamber assay, MASMs were starved overnight, suspended, and seeded (50 μL containing 5.0×10^4 cells) in the upper chamber on collagen-precoated PVP-free polycarbonate membrane. Control medium (DMEM/0.1% BSA) or CM 30 μL was placed in the lower chamber. After incubation at 37°C and 95% air/5% CO_2 for 8 hours, cells attached to the lower side were fixed in 4% formaldehyde/0.1% glutaraldehyde in 0.1 phosphate buffer, pH 7.2, and then stained for 30 minutes in 0.1% cresyl violet. The relative increases in cell number were determined by quantitative densitometry (ImagePro Plus).

Statistical Analysis

Quantitative results are expressed as mean±SD. Comparisons of parameters between 2 groups were made by unpaired Student's t test. Comparisons of parameters among the 3 groups were made by 1-way ANOVA, and comparisons of different parameters between the 2 genotypes were made by 2-way ANOVA followed by a post hoc analysis with the Bonferroni test. Statistical significance was evaluated with StatView (StatView 5.0, SAS Institute Inc, Cary, NC). A value of $P<0.05$ was considered statistically significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

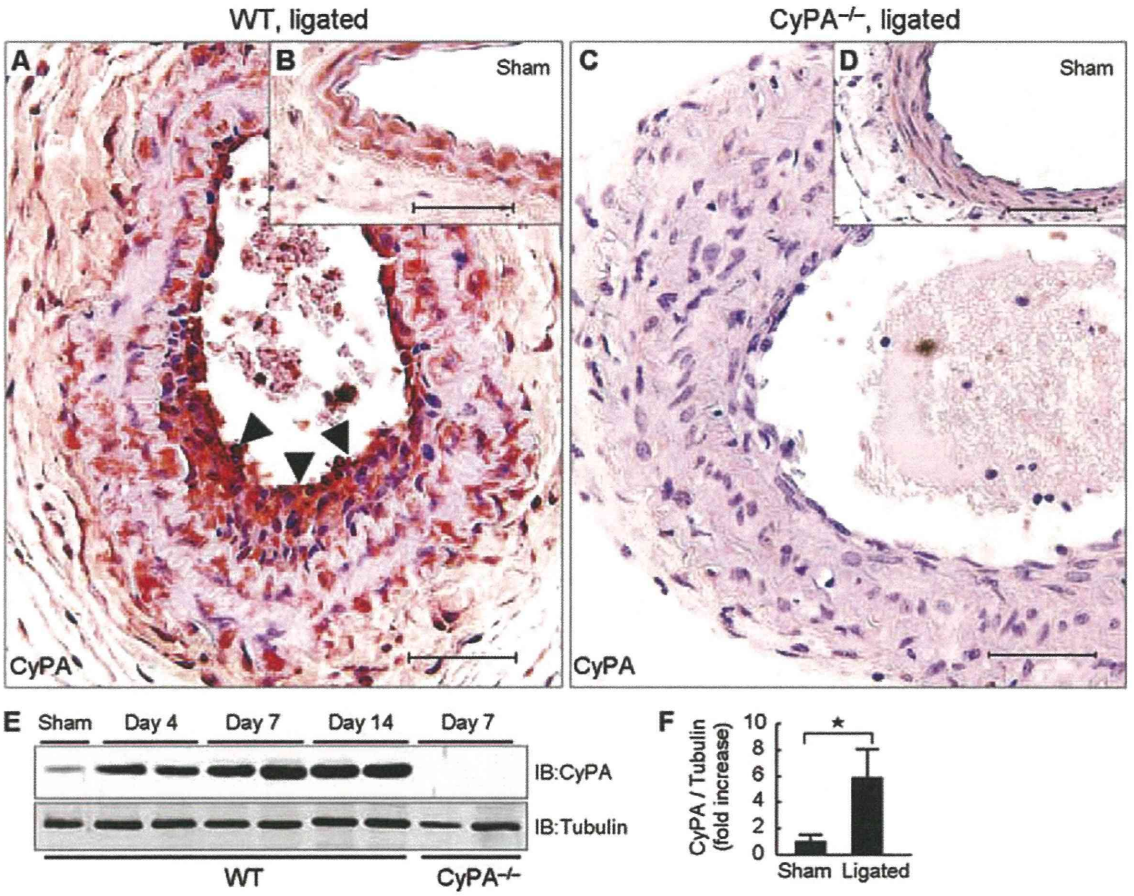

Figure 1. Carotid ligation enhances local CyPA expression. Representative immunostaining of CyPA in ligated (A and C) and sham (B and D) carotids from WT and CyPA$^{-/-}$ mice. Arrowheads show strong expression of CyPA in the intima. Scale bars=50 μm. E and F, Western blot analysis of CyPA in carotid homogenates of WT (n=8, solid bars) after ligation. Equal protein loading was confirmed with tubulin. Results are mean±SD. *P<0.01. IB indicates immunoblot.

Results

Vascular Remodeling After Carotid Ligation Was Significantly Reduced in CyPA$^{-/-}$ Mice

To test the hypothesis that CyPA contributes to vascular remodeling, we performed complete carotid ligation in WT and CyPA$^{-/-}$ mice. ROS generation and the proliferation of VSMCs play significant roles in intima formation in this model.[25,26] Immunostaining and Western blotting revealed that expression of CyPA increased after carotid ligation in WT mice, with highest expression in the intima (Figure 1A, arrows). In arteries that underwent a sham procedure, no increase in CyPA expression or intima formation was observed (Figure 1B). Quantification by Western blot showed a significant increase in CyPA expression after ligation in WT mice (Figure 1E and 1F). In CyPA$^{-/-}$ mice, there was no CyPA expression in either sham or ligated vessels (Figure 1C and 1D). These results indicate that blood flow cessation increases local CyPA expression. In sham WT and sham CyPA$^{-/-}$ controls, there was no intimal hyperplasia (Figure 2A and 2D), no differences in the thickness of elastic lamina (Figure 2B and 2E), similar α-SMA expression (Figure 2C and 2F), and no differences in medial area (Figure 2M, 2N, and 2O). The ligated arteries of WT mice (14 days) exhibited lumen narrowing (Figure 2G, 2H, and 2I), which was primarily a result of intimal hyperplasia and medial thickening. These changes were obviously reduced in CyPA$^{-/-}$ mice (Figure 2J, 2K, and 2L). Morphometric analysis of sham controls (Figure 2M, 2N, and 2O) showed no differences in intima, media, or intima/media (I/M) ratio in CyPA$^{-/-}$ mice versus WT mice. In contrast, analysis of ligated arteries on day 14 revealed that the intimal area was significantly smaller in CyPA$^{-/-}$ mice than in WT mice (Figure 2M). Additionally, we observed significantly increased medial thickening in WT mice compared with CyPA$^{-/-}$ mice (Figure 2N), which suggests that CyPA plays a crucial role in the development of vascular remodeling after blood flow cessation. Note that there was an increase in intimal area of CyPA$^{-/-}$ ligated vessels (Figure 2K and 2M) compared with sham, which indicates that other mechanisms also contribute to vascular remodeling. However, the I/M ratio of CyPA$^{-/-}$ mice was significantly less than that of WT mice (0.16±0.06 versus 0.29±0.13, P<0.01; Figure 2O), which indicates a crucial role of CyPA in vascular remodeling.

CyPA Induces VCAM-1 Expression and Promotes Inflammatory Cell Migration

An important initial step for the development of vascular lesions is inflammatory cell recruitment to the vascular wall.[7–9] Because CyPA induces VCAM-1 in human umbilical

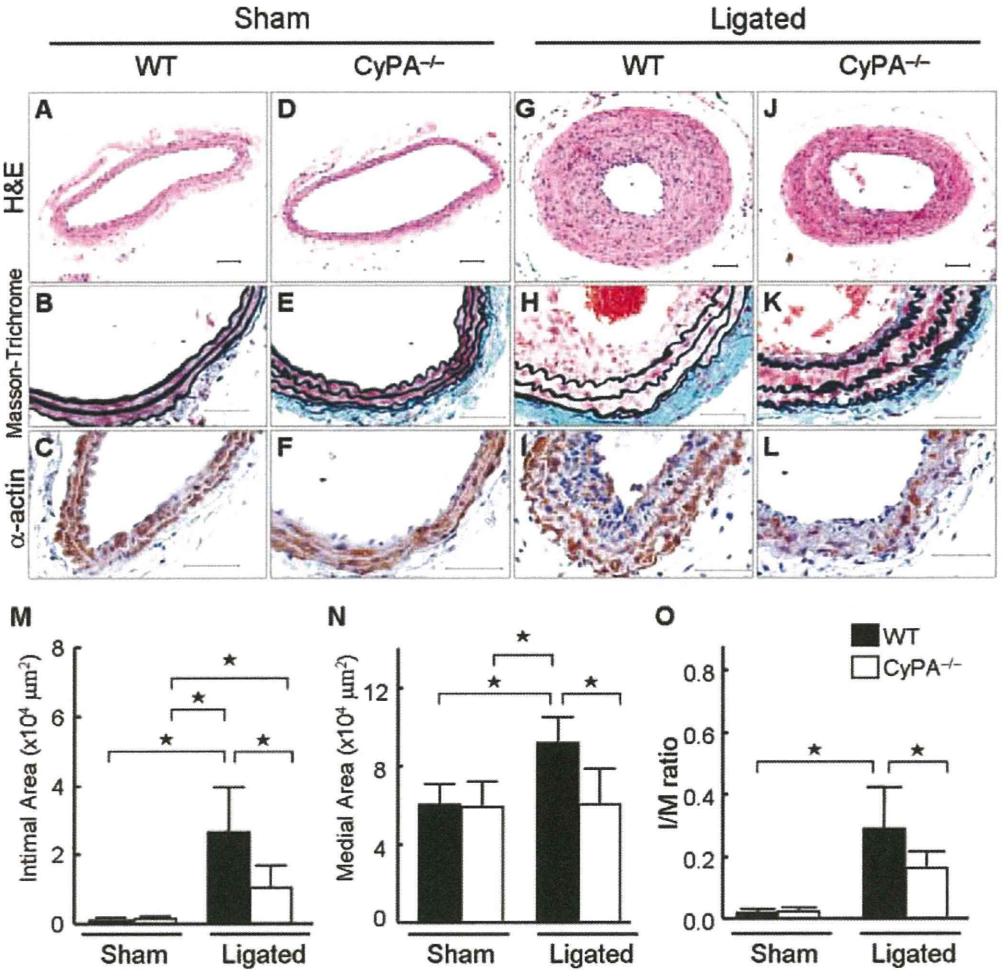

Figure 2. CyPA expression correlates with vascular remodeling. A–F, Photomicrographs showing representative cross-sectional areas of sham carotids of WT and CyPA$^{-/-}$ mice. The predominant cellular component in the intima was VSMC, as revealed by immunostaining for α-SMA. Scale bars=50 μm. G–L, Photomicrographs showing representative cross-sectional areas of ligated carotids of WT and CyPA$^{-/-}$ mice. CyPA deficiency significantly reduced intimal area (M), medial area (N), and I/M ratio (O) in CyPA$^{-/-}$ mice (n=8, open bars) compared with WT mice (n=9, solid bars). Results are mean±SD. *P<0.01. H&E indicates hematoxylin and eosin.

vein ECs[13] and is chemotactic for inflammatory cells,[14,15] we anticipated differences in inflammation based on CyPA expression. Expression of VCAM-1 was greatly increased in ligated WT carotids compared with sham (Figure 3A, 3B, and 3E). There was no difference in VCAM-1 expression in the sham carotids of WT versus CyPA$^{-/-}$ mice (Figure 3E). In contrast, expression of VCAM-1 was much lower in ligated carotids of CyPA$^{-/-}$ mice than in WT mice (Figure 3C, 3D, and 3E). The number of CD45^{+} inflammatory cells did not differ in sham arteries from WT versus CyPA$^{-/-}$ mice (Figure 3G and 3I). There was a significant increase in CD45^{+} cells in ligated arteries of WT mice (Figure 3F and 3G), whereas there was only a small change in CyPA$^{-/-}$ mice (Figure 3H and 3I). Quantification of CD45^{+} cells per intimal or medial area showed a 2-fold greater number in WT than in CyPA$^{-/-}$ mice (Figure 3J). These data suggest a role for CyPA in expression of VCAM-1 and inflammatory cell migration after carotid ligation.

Generation of VSMC-Specific CyPA Transgenic Mice

To test the hypothesis that VSMC-derived CyPA plays a major role in vascular remodeling, we prepared VSMC-Tg mice that overexpressed a FLAG-tagged CyPA only in VSMCs using a Cre/LoxP system. In brief, a LacZ^{flox}-CyPA construct (Data Supplement Figure I) was prepared with the pZ/EG vector, and LacZ^{flox}-CyPA mice were crossed with SM22α-Cre mice to achieve VSMC-specific expression. The vessels in Figure 4A through 4C are from control mice (LacZ^{flox}-CyPA^{+}/SM22α-Cre^{-}) in which the transgene expressed is LacZ, as shown by the blue β-gal staining and the low level of CyPA expression. Figure 4E shows the efficiency of Cre recombinase–mediated excision in vivo after mice were bred with SM22α-Cre to express FLAG-CyPA (SM22α-Cre×LacZ^{flox}-CyPA). Specifically, there was no LacZ expressed, as shown by β-gal staining, whereas anti-FLAG revealed significant FLAG-CyPA expression. The increase in CyPA was confirmed by anti-CyPA antibody (Figure 4F). To quantify FLAG-CyPA expression, we performed Western blots with anti-FLAG and anti-CyPA antibody. As shown in Figure 4G, the relative expression of exogenous CyPA (FLAG tagged) was 2.0-fold greater (in aorta) than that of endogenous CyPA. These experiments show that excision by SM22α-Cre was highly efficient (>90% of cells expressed FLAG, and <10% expressed

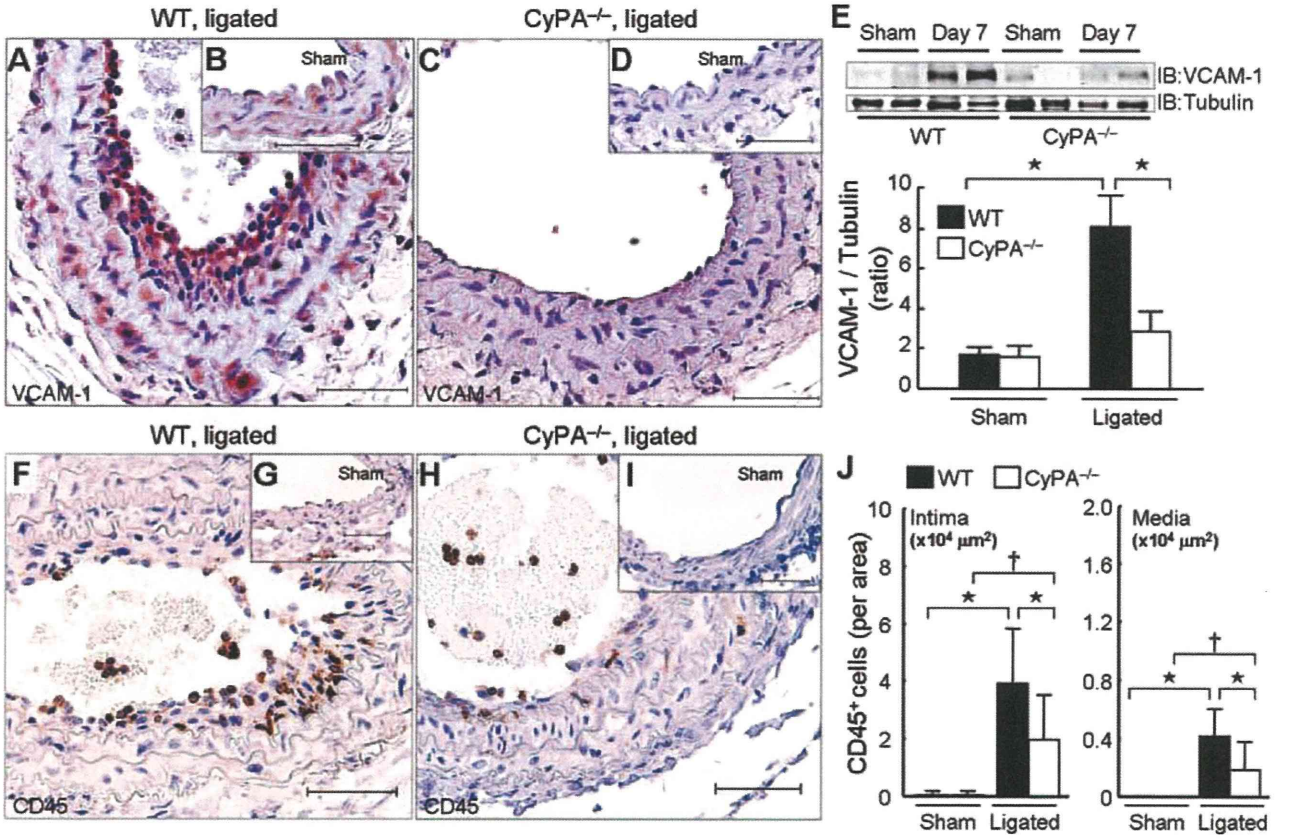

Figure 3. CyPA expression correlated with VCAM-1 expression and inflammatory cell accumulation. Representative immunostaining of VCAM-1 (A–D) and CD45 (F–I) in sham and ligated carotids from WT and CyPA$^{-/-}$ mice. Scale bars=50 μm. E, Western blot comparison of VCAM-1 in carotid homogenates of WT (n=8, solid bars) and CyPA$^{-/-}$ mice (n=6, open bars) at 7 days after ligation. Equal protein loading was confirmed with tubulin. J, The number of CD45^{+} cells in intima and media is increased by ligation in WT mice (n=9, solid bars) and was significantly less in CyPA$^{-/-}$ mice (n=8, open bars). Results are mean±SD. *P<0.01; †P<0.05. IB indicates immunoblot.

LacZ), and the expression of exogenous FLAG-CyPA was 2-fold greater than that of endogenous CyPA. To show that FLAG-CyPA was secreted similarly to endogenous CyPA, we stimulated VSMCs harvested from aorta of WT, CyPA$^{-/-}$, and VSMC-Tg mice with LY83583. LY83583 is a naphthoquinolinedione that undergoes futile redox cycling–generating intracellular ROS.[10,11] As expected, both FLAG-CyPA and endogenous CyPA were secreted in response to ROS in MASMs from VSMC-Tg aorta (Figure 4H). The magnitude of secreted FLAG-CyPA was equivalent to that of endogenous CyPA, similar to the expression of CyPA in lysates of intact aorta (Figure 4G).

VSMC-Specific CyPA Transgenic Mice Exhibit Dramatic Intimal and Medial Thickening

To prove further that VSMC-derived CyPA promotes vascular remodeling, we performed complete carotid ligation in VSMC-Tg (LacZ^{flox}-CyPA^{+}/SM22α-Cre^{+}) and control (LacZ^{flox}-CyPA^{+}/SM22α-Cre^{-}) mice. In sham arteries, intimal thickening was not observed in VSMC-Tg and control mice (Figure 5A through 5F), and the medial area did not differ significantly (Figure 5M and 5N). Two weeks after carotid ligation, intimal thickness was significantly increased in VSMC-Tg mice to a much greater extent than in control mice (Figure 5G through 5M). Additionally, we observed significantly increased medial thickening in VSMC-Tg mice (Figure 5N). The increased intima formation was due to VSMCs, as revealed by immunostaining for α-SMA (Figure 5H and 5I). The I/M ratio was increased 2.5-fold in VSMC-Tg, which suggests a pathogenic role for VSMC-derived CyPA in accumulation of VSMCs during vascular remodeling (Figure 5O). Inflammatory cell accumulation in the remodeled carotid wall was also increased significantly in VSMC-Tg mice (Figure 6), which suggests that VSMC-derived CyPA recruits inflammatory cells.

CyPA Plays a Crucial Role in VSMC Proliferation In Vivo

To strengthen the link between CyPA expression and VSMC growth, we carefully evaluated proliferation of VSMC by immunostaining for α-SMA, Ki67, and ERK1/2 phosphorylation (pERK1/2) on serial sections. We previously reported that ERK1/2 phosphorylation is important for VSMC migration and growth.[23] There was no difference in pERK1/2 expression (Data Supplement Figure IV, B and D) or Ki67^{+} VSMCs in sham carotids (Figure 7B and 7D); however, immunostaining and Western blotting revealed that pERK1/2 increased after carotid ligation in WT mice, with the highest expression in the intima compared with CyPA$^{-/-}$ carotids (Data Supplement Figure IV, A, C, E, and F). Consistently,

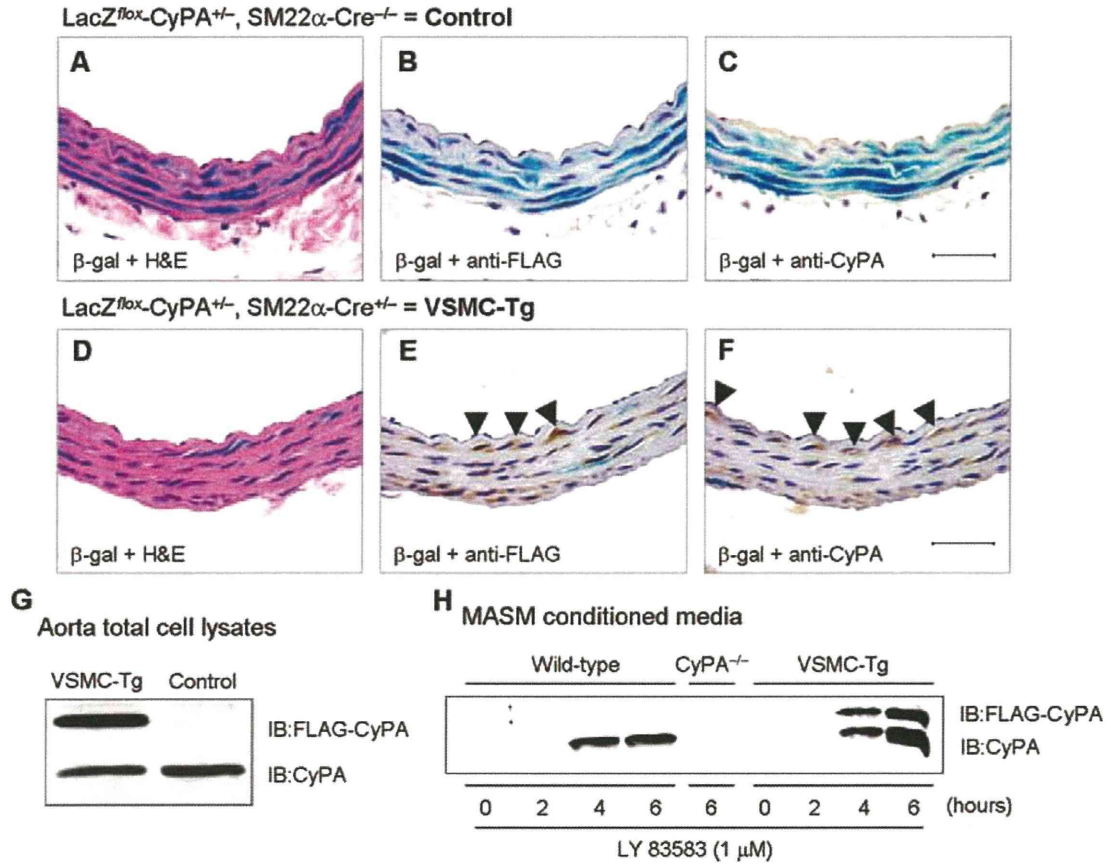

Figure 4. Characterization of CyPA expression in VSMC-Tg mice. A–F, Representative immunostaining and β-gal staining of aorta from VSMC-Tg mice (LacZ^{flox}-CyPA^{+}/SM22α-Cre^{+}) and control mice (LacZ^{flox}-CyPA^{+}/SM22α-Cre^{-}). β-Gal staining shows >90% expression only in VSMCs (B) and complete excision with SM22α-Cre (E). FLAG-CyPA was expressed only in VSMC-Tg mice and not in control mice. There was no change in endogenous CyPA expression. Scale bars=50 μm. G, Western blot demonstrated that FLAG-CyPA was expressed only in VSMC-Tg mice aorta, not in control mice. H, MASMs were stimulated with 1 μmol/L LY83583 for the indicated times, CM were prepared, and secreted CyPA and FLAG-CyPA were detected by Western blotting. IB indicates immunoblot; H&E, hematoxylin and eosin.

Ki67^{+} VSMCs were significantly increased in ligated WT carotids compared with CyPA$^{-/-}$ carotids (Figure 7A, 7C, and 7E). VSMC proliferation was even further enhanced in VSMC-Tg carotids compared with control carotids (Figure 7F through 7J), which suggests that CyPA promotes VSMC proliferation in vivo.

CyPA Plays a Crucial Role in Migration, Chemotaxis, and Proliferation of VSMCs In Vitro

To further confirm the role of CyPA in VSMC proliferation and migration, we harvested MASMs from the 3 mice strains and evaluated their proliferation and migration. To evaluate the effect of CyPA on VSMC migration and chemotaxis, we performed scratch-wound and Boyden chamber assays. The scratch wound was performed with WT-MASM as the "responder" cells and CM from the 3 strains. Tg-CM stimulated migration more than control-CM, and WT-CM stimulated migration more than KO-CM, which suggests that CyPA secreted into CM increased VSMC migration (Data Supplement Figure V). To measure the effect of CyPA on VSMC chemotaxis, we studied migration in response to serum and CM (Figure 8A and 8B). As anticipated, chemotaxis of KO-MASM was significantly reduced compared with WT-MASM and Tg-MASM in response to 10% serum, which suggests a role for intracellular CyPA in chemotaxis (Figure 8A). Next, we compared the chemotactic activity of CM from the 3 strains using WT-MASM as reporter cells. Migration of WT-MASM in response to Tg-CM was significantly increased compared with WT-CM (Figure 8B) and was dramatically greater than migration induced by KO-CM. These results indicate that secreted CyPA strongly enhances VSMC chemotaxis.

To determine the effect of varying CyPA secretion on VSMC growth, we measured the effects of CM on cell growth. Proliferation of WT-MASM in response to CM from Tg-MASM was significantly greater than that in response to CM from control-MASM (Figure 8C), which suggests that extracellular CyPA promotes VSMC growth. To assess the effect of CyPA expression on cell growth, we studied the ability of MASM from different strains to respond to both platelet-derived growth factor and serum (Figure 8D, 8E, and 8F). In the absence of exogenous growth stimuli, there was no difference in growth of cells isolated from VSMC-Tg compared with control (Figure 8D); however, growth in response to platelet-derived growth factor-BB and 10% serum was significantly increased in VSMC-Tg compared with control (Figure 8E and 8F). These data suggest that the level of CyPA expression has powerful effects on VSMC proliferation.

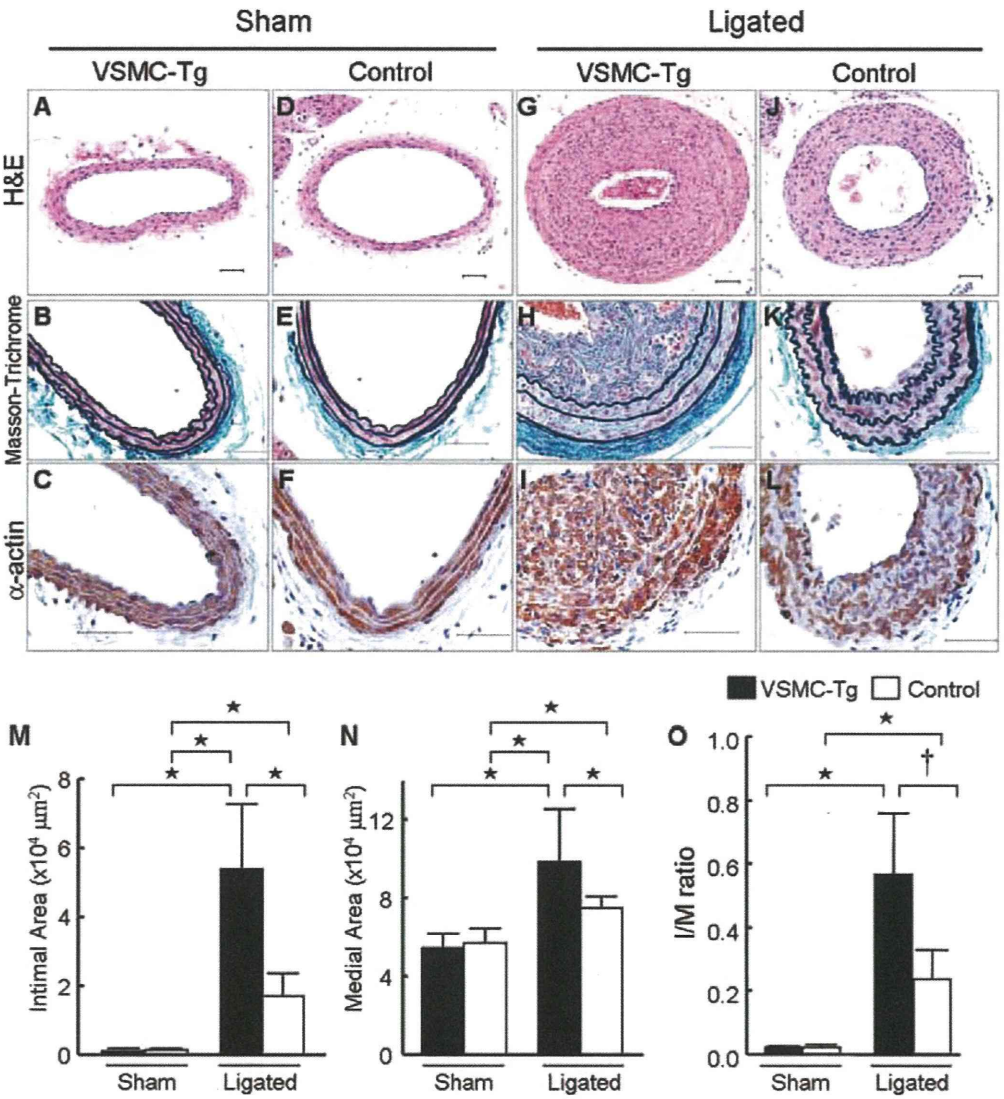

Figure 5. VSMC-specific overexpressed CyPA increases vascular remodeling after carotid ligation. Photomicrographs from sham (A–F) and ligated (G–L) mice carotids of VSMC-Tg and control. The predominant cellular component in the intima was VSMC, as revealed by immunostaining for α-SMA (α-actin). H&E indicates hematoxylin and eosin staining. Scale bars=50 μm. Intima (M), media (N), and adventitia area (O) in VSMC-Tg mice (n=7, solid bars) were greater than in control mice (n=6, open bars). Results are mean±SD. *$P<0.01$; †$P<0.05$.

Discussion

The major findings of the present study are that carotid ligation increases CyPA expression in the vascular wall and promotes vascular remodeling due to proliferation and migration of VSMCs and accumulation of inflammatory cells. These results are the first direct demonstration that CyPA contributes to vascular remodeling in vivo. The present study revealed 3 important pathological consequences of CyPA activity (Data Supplement Figure VI). First, VSMC-derived secreted CyPA increases intimal VSMCs by virtue of its ability to promote VSMC proliferation and migration. Second, secreted extracellular CyPA is proinflammatory, because it stimulates vascular expression of VCAM-1 and recruits inflammatory cells. Third, we showed a direct role for intracellular ROS to stimulate CyPA secretion that was proportionate to intracellular CyPA expression. These data show a role for CyPA as one of the key mediators of the pathological effects of ROS on vascular remodeling.

To strengthen the link between flow cessation, CyPA expression, and cell growth, we observed the time course and distribution of CyPA expression in carotids after ligation. There was minimal staining of CyPA in sham carotids but a dramatic increase in the intima and media after ligation. In parallel with CyPA expression, carotid ligation induced phosphorylation of ERK1/2 in WT carotids, which was significantly less in $CyPA^{-/-}$ carotids, consistent with the reduced number of $Ki67^+$ VSMCs in ligated $CyPA^{-/-}$ carotids. The distribution of $Ki67^+$ cells closely overlapped with areas of highest CyPA expression, especially in the rapidly proliferating intimal cells in WT mice (Data Supplement Figure III). Colocalization of CyPA and α-SMA staining revealed that CyPA expression was particularly high in VSMCs (Data Supplement Figure III). To further prove the contribution of VSMC-derived CyPA to vascular remodeling, we prepared VSMC-specific CyPA transgenic mice (VSMC-Tg). VSMC-Tg mice exhibited no significant change in sham

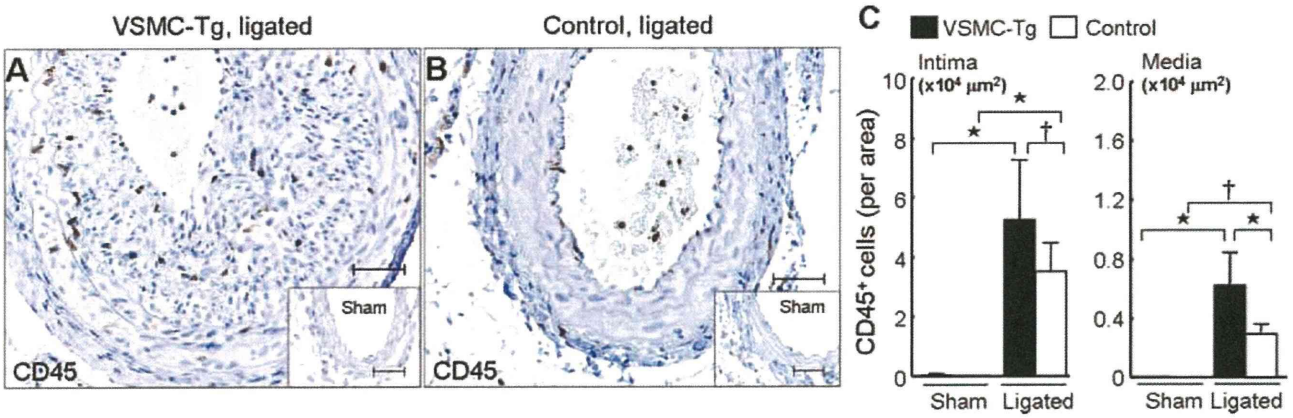

Figure 6. Overexpression of CyPA promotes recruitment of inflammatory cells in ligated carotids. Representative immunostaining of CD45 in ligated arteries from VSMC-Tg (A) and control mice (B). C, Number of CD45$^+$ cells in VSMC-Tg (n=7, solid bars) and control mice (n=6, open bars). VSMC-specific overexpression of CyPA enhanced the recruitment of CD45$^+$ cells to the intima and media. Results are mean±SD. *P<0.01; †P<0.05.

carotids, whereas ligated carotids showed increases of 217% in intimal area, 32% in medial area, and 140% in I/M ratio compared with control mice expressing normal levels of CyPA. The observation that VSMC-specific CyPA overexpression increased not only the medial area but also the intimal area suggests that VSMC-derived extracellular CyPA promotes the proliferation and migration of VSMCs via a secreted, paracrine pathway. VSMC proliferation measured

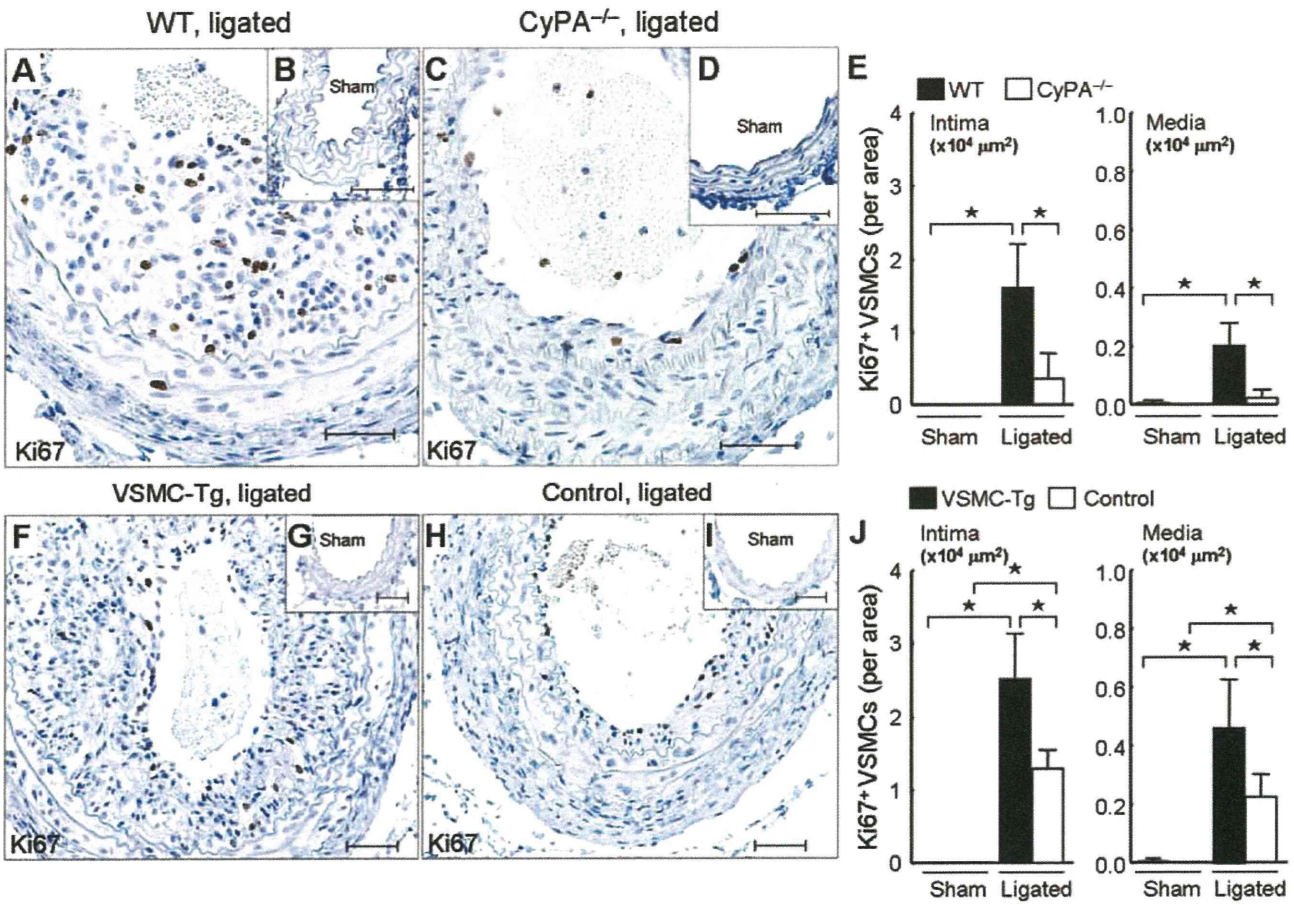

Figure 7. CyPA increases VSMC proliferation in ligated carotids. Representative immunostaining of Ki67 and counterstaining with hematoxylin in sham carotids and ligated carotids from WT, CyPA$^{-/-}$, VSMC-Tg, and control mice. A–E, There were a significantly increased number of Ki67$^+$ cells in ligated WT carotids (n=9, solid bars) compared with CyPA$^{-/-}$ carotids (n=8, open bars). Scale bars=50 μm. E, Analysis of numbers of α-SMA$^+$ Ki67$^+$ cells by staining of serial section (3 sections per mouse) was performed. F–J, There was a significant increase in Ki67$^+$ VSMCs in ligated VSMC-Tg carotids (n=7, solid bars) compared with control carotids (n=6, open bars). J, Analysis of numbers of α-SMA$^+$ Ki67$^+$ cells by staining of serial section (3 sections per mouse) was performed. Results are mean±SD. *P<0.01.

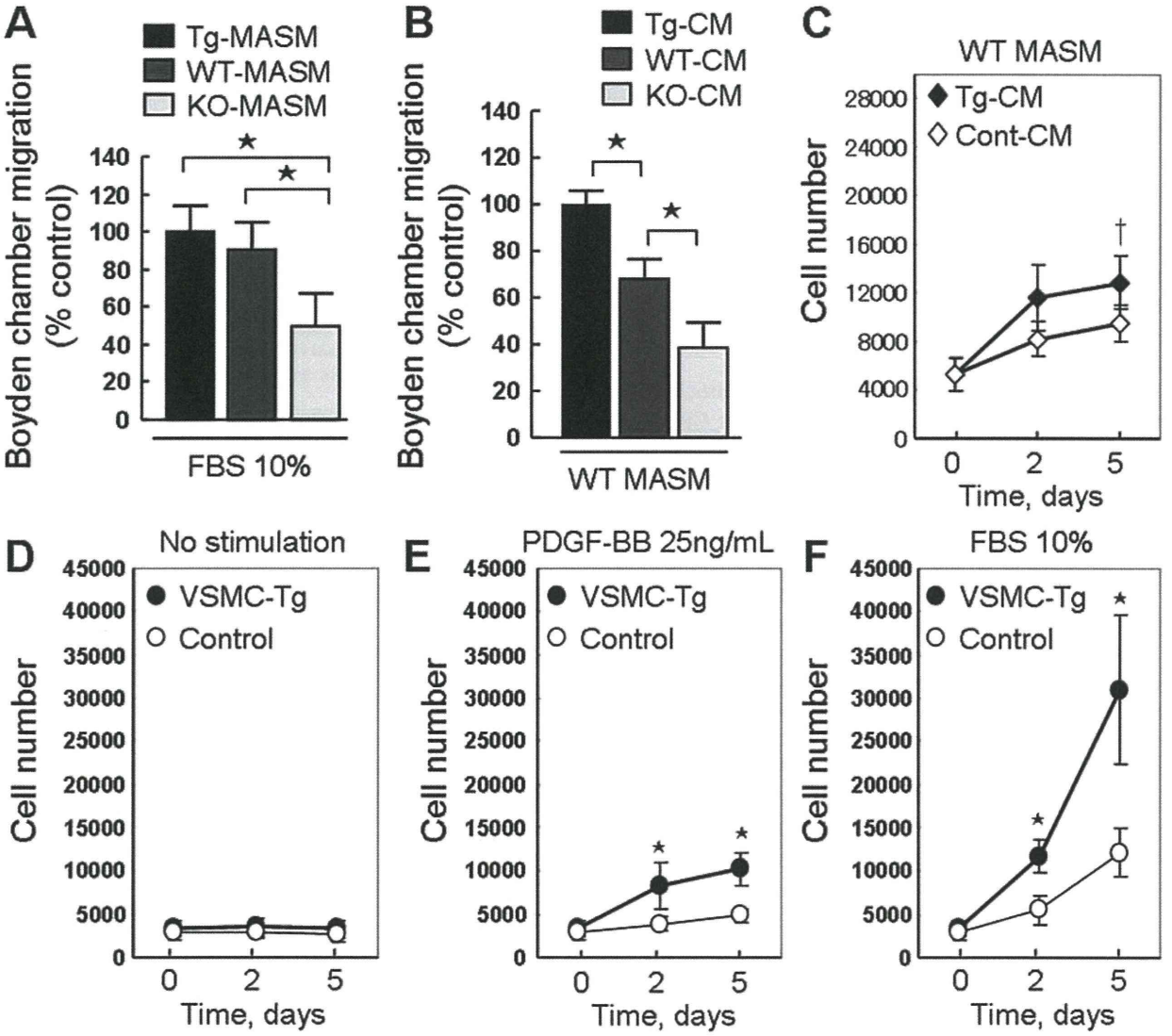

Figure 8. CyPA promotes migration and proliferation of MASMs. A, Migration of MASMs in response to 10% fetal bovine serum (FBS) in Boyden chamber assay. Tg-MASMs, WT-MASMs, and KO-MASMs were starved overnight and then seeded in the upper Boyden chamber on collagen-precoated PVP-free polycarbonate membranes. FBS (10%) was added to the lower chamber. Migration was determined, and the maximum increase was normalized to 100%. B, WT-MASMs were starved overnight and then seeded in the upper Boyden chamber. Tg-CM, WT-CM, or KO-CM was added to the lower chamber. Cells were incubated for 8 hours at 37°C in a 95% air/5% CO_2 humidified incubator. The membranes were removed, and cells were stained. The relative increases in cell number were determined by quantitative densitometry. *$P<0.01$. Data are mean±SD; n=6 in each group. C, CM from VSMC-Tg (Tg-CM) or control (Cont-CM) promotes cell proliferation. WT-MASMs were seeded in 96-well plates in DMEM supplemented with 10% FBS, serum starved for 24 hours, and stimulated with Tg-CM or Cont-CM for 5 days. CM was changed at day 3, and cells were counted at day 2 and day 5. Data are mean±SD. †$P<0.05$. D–F, Effect of platelet-derived growth factor-BB (PDGF-BB) and FBS on proliferation of Tg-MASMs and control MASMs. After starvation for 24 hours, MASMs were incubated with DMEM (D) or stimulated with 25 ng/mL PDGF-BB (E) or 10% FBS (F) for 5 days. Medium was changed at day 3, and cells were counted at day 2 and day 5. Data are mean±SD. *$P<0.01$. n=8 in each group.

by Ki67 correlated significantly with CyPA expression (VSMC-Tg>control WT>CyPA$^{-/-}$). VSMC migration and chemotaxis similarly correlated with the magnitude of CyPA expression. The increased VSMC proliferation and migration that resulted from VSMC-specific overexpression of CyPA suggests a major contribution for VSMC-derived CyPA in vascular remodeling.

Turbulent blood flow and reduced shear stress generate ROS and play a crucial role in the development of atherosclerosis due to local inflammation.[7–9] In VSMCs, ROS activate a pathway that induces secretion of CyPA,[12] which stimulates at least 3 signaling pathways (ERK1/2, Akt, and JAK).[10] Extracellular CyPA activates proinflammatory pathways in ECs, including increased expression of VCAM-1.[13] Additionally, CyPA itself is a chemoattractant and promotes migration of several cell types in vitro.[13–15] Consistently, carotid ligation increased VCAM-1 expression in ligated WT carotids. VCAM-1 expression was significantly less in ligated CyPA$^{-/-}$ carotids, which corresponded to reduced accumulation of CD45^{+} cells in the intima. This implies that the decreased accumulation of inflammatory cells in CyPA$^{-/-}$ carotids likely results from reduced expression of VCAM-1

and decreased chemotactic signaling in the absence of CyPA. Because decreased myelopoiesis could also explain diminished inflammatory cell recruitment in CyPA$^{-/-}$ mice, we analyzed white blood cell counts from mice with WT or CyPA$^{-/-}$ bone marrow. The number of circulating monocytes was not altered by CyPA deficiency or by carotid ligation. Thus, lack of CyPA in bone marrow cells does not alter inflammatory cell production or ability to enter the peripheral circulation. In contrast to the situation in CyPA$^{-/-}$ mice, VSMC-specific overexpression of CyPA (VSMC-Tg) further enhanced the accumulation of inflammatory cells in ligated carotids, which supports the important role for CyPA in mediating the recruitment of inflammatory cells.

CyPA is expressed by all cell types that participate in vascular pathology.[25] Additionally, extracellular CyPA has recently been found to induce interleukin-6 release in inflammatory cells.[27] We observed significant accumulation of inflammatory cells in the intima. We propose that ROS generated locally by inflammatory cells causes VSMCs to release CyPA, which would promote a proinflammatory cycle for vascular remodeling. Therefore, in addition to VSMC-derived CyPA, the contribution of inflammatory cells is important for intima formation in this model. Recent observations support the contribution of inflammatory cells to intimal thickening.[28,29] CyPA could regulate the proteolytic activity necessary for the migration of inflammatory cells through activating MMPs, especially MMP-1 and MMP-9.[14,16] Considering the importance of migrating inflammatory cells in vascular remodeling,[30,31] local cytokine production by inflammatory cells could promote intima formation in this model.

Study Limitations

CyPA is a chaperone protein that is widely expressed, including inflammatory cells and ECs. Therefore, CyPA in macrophages and ECs may play an important role in vascular remodeling. Future studies will be required to define the role of CyPA in the response to vascular injury of cells such as macrophages, T and B cells, mast cells, platelets, and vascular progenitor cells.

Clinical Implications and Conclusions

The present data from the use of genetically engineered mice to modulate vascular CyPA expression prove that a decrease in CyPA levels has beneficial effects on the inflammatory response and vascular intima formation, as shown by significantly increased lumen diameter and decreased I/M ratio. This is consistent with our findings that the plasma level of CyPA is increased in patients with acute coronary syndromes.[13] Additionally, Billich et al[32] reported increased concentrations of CyPA in synovial fluids of patients with rheumatoid arthritis. These results suggest that extracellular CyPA is a novel mediator for vascular disease associated with ROS and inflammation.

Acknowledgments

We are grateful to the Aab Cardiovascular Research Institute members for useful suggestions and Chris Ashley, Robert Winterkorn, Mary A. Georger, Tamlyn Thomas, and Sarah A. Mack for technical assistance.

Sources of Funding

This work was supported by National Institutes of Health grant HL49192 (to Dr Berk) and a Japan Heart Foundation/Bayer Yakuhin research grant abroad (to Dr Satoh).

Disclosures

None.

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CLINICAL PERSPECTIVE

Decreased blood flow distal to a stenosis is associated with accelerated atherosclerosis and occlusion, but the mechanisms are not fully elucidated. Accumulating evidence indicates that inflammation and vascular smooth muscle cell (VSMC) proliferation contributes to vessel narrowing. It has become clear that an increase in reactive oxygen species is a key pathogenic mechanism for vascular disease. Cyclophilin A (CyPA) is a 20-kDa chaperone protein secreted from VSMCs in response to reactive oxygen species that stimulates VSMC proliferation and inflammatory cell migration in vitro. Here, using genetically engineered mice to modulate vascular CyPA expression, we show that decreasing CyPA has beneficial effects on the inflammatory response and vascular intima formation in low-flow vessels, as shown by significantly increased lumen diameter and decreased intima/media ratio. The present study may have important clinical implications, because it appears that secreted CyPA mediates the growth and inflammation observed in low-flow vessels. This suggests that a receptor for CyPA may represent an attractive therapeutic target for vascular diseases associated with oxidative stress and inflammation.

Cardiovascular Research (2009) 81, 226–234
doi:10.1093/cvr/cvn244

Statin ameliorates hypoxia-induced pulmonary hypertension associated with down-regulated stromal cell-derived factor-1

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Received 21 July 2008; revised 28 August 2008; accepted 4 September 2008; online publish-ahead-of-print 8 September 2008

Time for primary review: 29 days

KEYWORDS
Statin;
Pulmonary hypertension;
Hypoxia;
Myofibroblast;
Progenitors

Aims Mobilization of stem cells/progenitors is regulated by the interaction between stromal cell-derived factor-1 (SDF-1) and its ligand, CXC chemokine receptor 4 (CXCR4). Statins have been suggested to ameliorate pulmonary arterial hypertension (PAH); however, the mechanisms involved, especially their effects on progenitors, are largely unknown. Therefore, we examined whether pravastatin ameliorates hypoxia-induced PAH in mice, and if so, which type of progenitors and what mechanism(s) are involved.

Methods and results Chronic hypoxia (10% O_2 for 5 weeks) increased the plasma levels of SDF-1 and mobilization of $CXCR4^+$/vascular endothelial growth factor receptor $(VEGFR)2^+/c\text{-}kit^+$ cells from bone marrow (BM) to pulmonary artery adventitia in Balb/c mice *in vivo*, both of which were significantly suppressed by simultaneous oral treatment with pravastatin (2 mg/kg/day). Furthermore, *in vitro* experiments demonstrated that hypoxia enhances differentiation of $VEGFR2^+/c\text{-}kit^+$ cells into α-smooth muscle $actin^+$ cells. Importantly, pravastatin ameliorated hypoxia-induced PAH associated with a decrease in the number of BM-derived progenitors accumulating in the pulmonary artery adventitia. The expression of intercellular adhesion molecule-1 (ICAM-1) and its ligand, CD18 (β2-integrin), were enhanced by hypoxia and were again suppressed by pravastatin.

Conclusions These results suggest that pravastatin ameliorates hypoxia-induced PAH through suppression of SDF-1/CXCR4 and ICAM-1/CD18 pathways with a resultant reduction in the mobilization and homing of BM-derived progenitor cells.

1. Introduction

Recent reports demonstrated that circulating endothelial progenitor cells (EPCs) promote endothelial repair, which is enhanced by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins).[1–3] Moreover, the number of circulating EPCs correlates with endothelial function and the degree of coronary artery disease in humans.[4] Accumulating evidence suggests that circulating EPCs are mobilized to the site of ischaemia by several humoral factors, such as vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1), CD18, and intercellular adhesion molecule-1 (ICAM-1), contributing to the neovascularization.[1,5,6] Recently, it has also been reported that CXC chemokine receptor 4 (CXCR4), the receptor for SDF-1, plays an important role in the mobilization and recruitment of bone marrow (BM)-derived cells.[7] We have recently reported that EPCs are mobilized under hypoxic conditions and are incorporated into the pulmonary endothelium in pulmonary arterial hypertension (PAH) in mice.[8] Recent studies also demonstrated the therapeutic effects of EPC transplantation in animal models of PAH.[9,10] However, it remains to be examined whether EPCs also exert beneficial effects in patients with PAH, which is characterized by plexiform lesion composed of actively proliferating endothelial cells.[11]

It has been demonstrated that statins could prevent the development of PAH in animal models.[12–14] Although statins could mobilize EPCs,[15,16] long-term statin treatment has been reported to reduce the number of circulating EPCs in patients with coronary artery disease.[17] Therefore,

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statins might have biphasic effects on mobilization of EPCs,[18] as well as other circulating progenitor cells, which have also been reported to exist and differentiate into α-smooth muscle actin (SMA)$^+$ cells in humans,[19,20] and may participate in the progression of atherosclerosis[21–23] and lung fibrosis.[24] Recent reports also showed the existence of circulating progenitors that exhibit fibroblast-like properties, migrate into the pulmonary vasculature, and promote adventitial remodelling as myofibroblasts (α-SMA-expressing fibroblasts) in hypoxia-induced PAH.[25–27] Indeed, the BM-derived progenitors could differentiate into α-SMA$^+$ cells in hypoxia-induced PAH in mice.[28] However, it largely remains unknown whether statins affect mobilization and recruitment of those progenitors.

In the present study, we thus examined whether pravastatin ameliorates hypoxia-induced PAH in mice, and if so, which type of progenitors and what mechanism(s) are involved.

2. Methods

All procedures were performed according to the protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 8523, revised 1985). The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the article as written.

2.1 Animal preparation

In the present study, we used 16-week-old wild-type (WT, $n = 56$) mice of Balb/c background. ROSA26 (LacZ) mice that express β-galactosidase (β-gal) activity in all tissues[29] were purchased from Jackson Laboratory (Bar Harbor, ME, USA).

2.2 Hypoxia-induced pulmonary arterial hypertension model in mice and statin treatment

The present study is a preventive study in nature. Four weeks after the BM transplantation, β-gal-BM chimeric mice were randomized to receive either pravastatin (2 mg/kg/day) or vehicle by daily gavage, and then exposed to hypoxia (10% O_2, for *in vivo* experiment) for 5 weeks in a hypoxic chamber, as previously described.[8,12] As a control, chimeric mice were maintained in plastic cages in ambient air (21% O_2). After 5 weeks of chronic hypoxia, control and hypoxic mice were anaesthetized with intraperitoneal ketamine hydrochloride (60 mg/kg) and xylazine (8 mg/kg) ($n = 10$, respectively), and right ventricular systolic pressure (RVSP) was measured by percutaneous insertion into the right ventricle (RV) through the subxiphoid approach of a 25-gauge needle connected to a pressure transducer without the use of respirator nor opening the chests. To evaluate the extent of RV hypertrophy, the RV free wall and left ventricle (LV) plus septum (LV+S) were weighed separately.

2.3 *Ex vivo* culture of mononuclear cells

To confirm the differentiation capacity, isolated peripheral blood mononuclear cells (PBMCs) from hypoxic mice (10% O_2 for 7 days) were cultured on collagen I-coated chamber slides (BioCoat; Becton Dickinson, San Jose, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in hypoxic conditions (2% O_2, 5% CO_2, and 93% nitrogen, 33°C, for *in vitro* experiment). For immunohistological staining, monoclonal antibody to fluorescein isothiocyanate (FITC)-labelled α-SMA (1:400, Sigma, St Louis, USA) was used for primary antibody.

2.4 Fluorescence-activated cell sorter analysis

Fluorescence-activated cell sorter (FACS) analysis was performed as described previously.[8,9] To quantify the number of VEGFR2$^+$/c-kit$^+$ cells, we used phycoerythrin-labelled anti-mouse VEGFR2, FITC-labelled anti-mouse c-kit (eBioscience), and biotinylated anti-mouse lineage antibodies (Mac-1, Gr-1, B220, CD4, CD8, and Ter119) and APC-labelled streptavidin (BD PharMingen). Quantitative analysis was performed by FACS analysis (FACSCalibur; Becton Dickinson).

2.5 Cell sorting by fluorescence-activated cell sorter

BM cells were obtained from hypoxic mice, and BM mononuclear cells were isolated by density gradient centrifugation with Nycoprep Animal 1.077 (Axis-Shield). Lin$^-$ /VEGFR2$^+$/c-kit$^+$ cells were selected with a cell sorting system (FACSAria; Becton Dickinson Immunocytometry Systems).[30]

2.6 Bone marrow transplantation

BM transplantation was performed as described previously.[8] The chimeric rate was >95% by FACS analysis.

2.7 Immunofluorescence staining

Immunofluorescence staining was performed on 4% of paraformaldehyde-fixed frozen sections as previously described.[8] The primary antibodies used were FITC-labelled anti-β-gal (1:400; Abcam Ltd, Cambridge, UK), anti-mouse ICAM-1 (1:400; BD PharMingen), anti-mouse CD18 (1:400; BD PharMingen), Cy3-labelled anti-α-SMA (1:400; Sigma), anti-VEGFR2 (1:200; Santa Cruz), anti-CD31, biotinylated anti-c-kit, and biotinylated anti-CXCR4 (1:400; BD PharMingen, San Diego). As the secondary antibodies, Cy3- or Cy5-labelled antibodies were used (Jackson ImmunoResearch Laboratories). Vectashield mounting medium with DAPI (vector) was used to counterstain nuclei. Slides were viewed with a confocal fluorescence microscope (Fluoview FV1000, Olympus, Tokyo, Japan). As a negative control, species- and isotype-matched IgG were used in place of the primary antibody.

2.8 X-gal staining

To detect β-gal-positive cells, the lungs were perfusion-fixed with 0.5% glutaraldehyde (pH 7.2) at 4°C and the whole lungs were incubated at 37°C for 18 h in X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) solution as previously described.[29]

2.9 Plasma levels of stromal-derived factor-1

Plasma levels of SDF-1 were evaluated by ELISA with a mouse SDF-1 Quantikine kit (R&D) following the manufacture's protocol.

2.10 Statistical analysis

Quantitative results are expressed as means ± SD. Statistical analysis was performed with StatView (StatView 5.0, SAS Institute Inc., Cary, NC, USA). Comparisons of parameters among the three groups were made by one-way ANOVA and those between the two groups under different conditions by two-way ANOVA followed by Bonferroni *post hoc* test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 Mobilization of vascular endothelial growth factor receptor 2$^+$/c-kit$^+$ cells in hypoxia-induced pulmonary arterial hypertension in mice

To elucidate the role of circulating VEGFR2$^+$/c-kit$^+$ cells, we performed FACS analysis on PBMCs from the mice that were

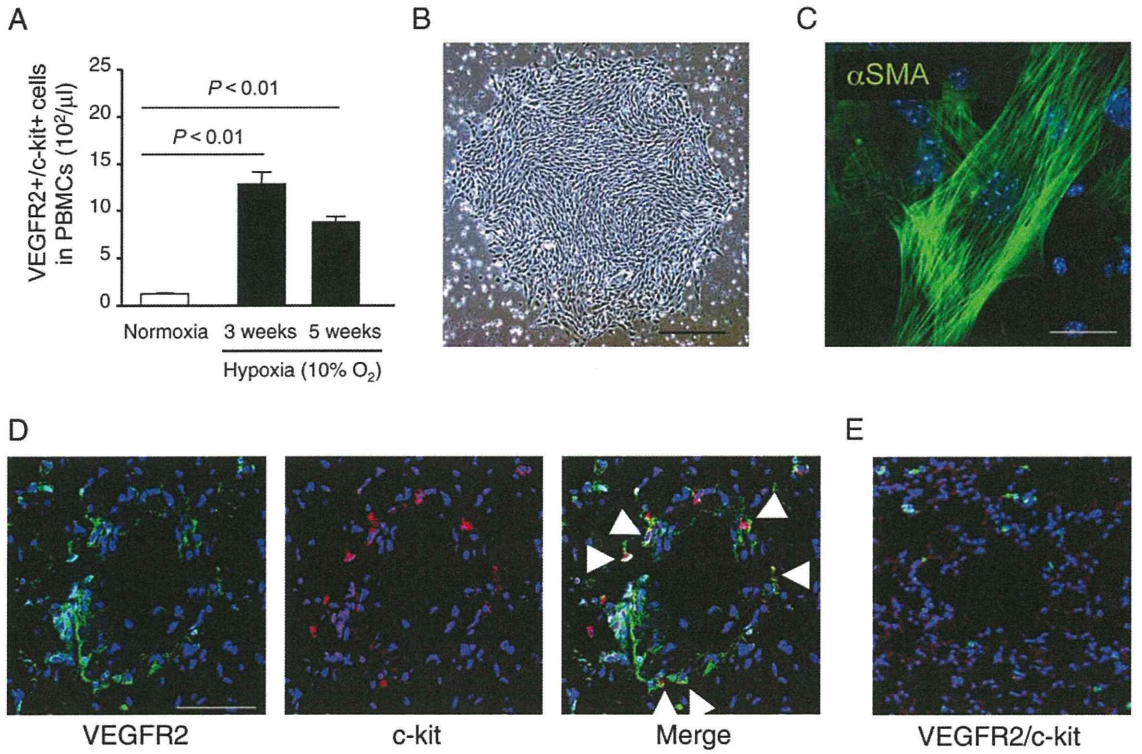

Figure 1 Vascular endothelial growth factor receptor (VEGFR)2^+/c-kit^+ cells to the adventitia of pulmonary arteries in hypoxia. (*A*) Fluorescence-activated cell sorter analysis indicated that hypoxia significantly increased the number of circulating VEGFR2^+/c-kit^+ cells at 3 and 5 weeks ($n = 6$ each). Results are expressed as means ± SD. (*B*) Colonies with a 'hill and valley' morphology after cultivation of peripheral blood mononuclear cells for 3 weeks under hypoxic conditions (2% O_2). Scale bar: 200 μm. (*C*) α-SMA immuno-positive cells in the colony. Scale bar: 10 μm. (*D,E*) VEGFR2^+/c-kit^+ cells in the adventitia of pulmonary arteries in mice with hypoxic condition (5 weeks) detected by immunostaining (*D*, arrows), but not in normoxic mice (*E*). Scale bar: 50 μm.

chronically exposed to hypoxia (10% O_2) for 5 weeks. Interestingly, the number of cells was significantly increased in hypoxic mice compared with that in normoxic mice at 3 and 5 weeks of hypoxia (*Figure 1A*). Moreover, cultivation of PBMCs from hypoxic mice revealed a colony formation (*Figure 1B*). Immunostaining revealed that these cells were positive for α-SMA (*Figure 1C*). Furthermore, VEGFR2^+/c-kit^+ cells were accumulated mainly at the adventitia of pulmonary arteries of chronically hypoxic mice (*Figure 1D*), whereas VEGFR2^+/c-kit^+ cells were not observed in normoxic condition (*Figure 1E*). These results suggest a crucial role of circulating VEGFR2^+/c-kit^+ cells in the pathogenesis of hypoxia-induced PAH.

3.2 Differentiation of vascular endothelial growth factor receptor 2^+/c-kit^+ cells into α-smooth muscle $actin^+$ cells

To elucidate the role of VEGFR2^+/c-kit^+ cells, we isolated VEGFR2^+/c-kit^+ cells from BM of Rosa26 mice by flow cytometry (*Figure 2A*) and cultured them on primary cultured stromal cell layers (derived from WT mice) under hypoxic conditions (2% O_2, 33°C) for 3 weeks (*Figure 2B*). Interestingly, these cells differentiated into α-SMA^+ cells after 3 weeks of culture (*Figure 2C*).

3.3 Pravastatin reduces the number of bone marrow-derived cells at the pulmonary artery adventitia

To elucidate the effects of pravastatin on the recruitment of the BM-derived cells and the development of pulmonary vascular remodelling, we used chimeric mice with β-gal-labelled BM. These chimeric mice were chronically exposed to hypoxia (10% O_2) for 5 weeks with or without simultaneous treatment with pravastatin (2 mg/kg/day). Microscopic observation revealed that the BM-derived (β-gal^+) cells migrated and accumulated to the adventitia of pulmonary arteries (*Figure 3A*) and that pravastatin significantly reduced the number of those cells (*Figure 3A* and *B*).

3.4 Pravastatin reduces mobilization of vascular endothelial growth factor receptor 2^+/c-kit^+ cells and ameliorates pulmonary arterial hypertension

It has been shown that the secretion of SDF-1 is controlled by hypoxia-induced factor-1α (HIF-1α), which have chemotactic power to mobilize the BM-derived progenitors.[31,32] We therefore assessed the plasma levels of SDF-1 in chronically hypoxic mice with or without pravastatin. Interestingly, plasma levels of SDF-1 were significantly increased in hypoxic mice, which was significantly reduced by pravastatin (*Figure 4A*). Moreover, immunostaining revealed that hypoxia enhanced the accumulation of $CXCR4^+$ cells in the lung (*Figure 4B*) and FACS analysis showed the hypoxia-induced increase in the number of circulating $CXCR4^+$/VEGFR2^+/c-kit^+ cells in peripheral blood (*Figure 4C*), both of which also were inhibited by pravastatin. Finally, we confirmed that pravastatin ameliorated PH in mice *in vivo*, as assessed by RVSP (*Figure 4D*) and RV hypertrophy (*Figure 4E*).

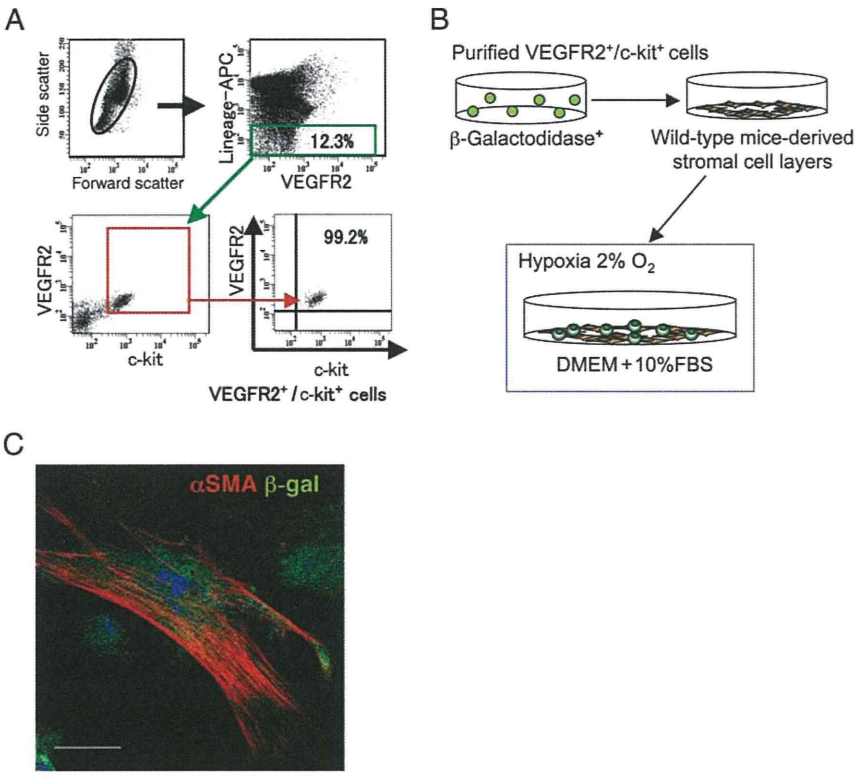

Figure 2 Differentiation of purified vascular endothelial growth factor receptor (VEGFR)2$^+$/c-kit$^+$ cells into α-smooth muscle actin (SMA)$^+$ cells under hypoxic conditions. (*A*) Purification from the bone marrow of ROSA26 mice by flow cytometry. (*B*) Cultivation on the irradiated stromal cell layers under hypoxic conditions for 3 weeks. (*C*) Differentiation of purified VEGFR2$^+$/c-kit$^+$ cells (β-galactosidase$^+$) into α-SMA$^+$ cells. Scale bar: 10 μm.

3.5 Pravastatin reduces accumulation of bone marrow-derived cells by suppressing ICAM-1 expression

It has been implicated that the interaction between ICAM-1 and its ligand, CD18, is crucial for the recruitment of the BM-derived progenitors.[33] We therefore assessed ICAM-1 expression in the hypoxic lung, the number of BM-derived cells, and their CD18 expression. Interestingly, hypoxia enhanced the expression of ICAM-1 in the lung, which was again suppressed by pravastatin (*Figure 5A*). Moreover, the BM-derived CD18$^+$ cells accumulated into the pulmonary artery adventitia in hypoxic lung, which was again reduced by pravastatin (*Figure 5A* and *B*).

4. Discussion

The novel findings of the present study were that: (i) chronic hypoxia significantly mobilized VEGFR2$^+$/c-kit$^+$ cells that accumulated into the pulmonary artery adventitia *in vivo* and differentiated into α-SMA$^+$ cells *in vitro* and (ii) pravastatin reduced the plasma levels of SDF-1, mobilization of CXCR4$^+$/VEGFR2$^+$/c-kit$^+$ cells, and the expression of ICAM-1 in the lung, resulting in the reduced accumulation of BM-derived progenitors and amelioration of PAH. The present findings are summarized in *Figure 6*. To the best of our knowledge, this is the first study that demonstrates the therapeutic effects of a statin for PAH in the cutting edge of circulating progenitors.

4.1 Circulating progenitors and pulmonary arterial hypertension

We have recently demonstrated that circulating progenitors are incorporated into the pulmonary endothelium and that mobilization and homing of progenitors are important in the pulmonary vascular remodelling as they exert beneficial effects in hypoxia-induced PAH in mice.[8] In the present study, circulating mononuclear cells isolated from mice chronically exposed to hypoxia differentiated into α-SMA$^+$ cells *in vitro*. Consistent with our finding, a recent report demonstrated that circulating c-kit$^+$ progenitor cells are involved in vessel wall thickening in hypoxia-induced PAH in calves.[25] Moreover, it has been shown that BM-derived cells differentiate into α-SMA$^+$ cells in pulmonary arteries in hypoxia-induced PAH in mice.[28] It has also been demonstrated that circulating progenitors are heterogeneous and that haematopoietic stem cell-derived progenitors contribute to neoangiogenesis through adventitial infiltration, without any contributions to pulmonary endothelium in PAH animal models.[34,35] Therefore, the BM-derived circulating progenitors may play a crucial role in the development of hypoxia-induced pulmonary vascular remodelling.

In the present study, we were also able to demonstrate the significant mobilization and accumulation of VEGFR2$^+$/c-kit$^+$ cells at the pulmonary artery adventitia *in vivo* and their differentiation into α-SMA$^+$ cells *ex vivo*. Recent reports demonstrated that α-SMA$^+$ cells at the pulmonary artery adventitia can be derived from circulating

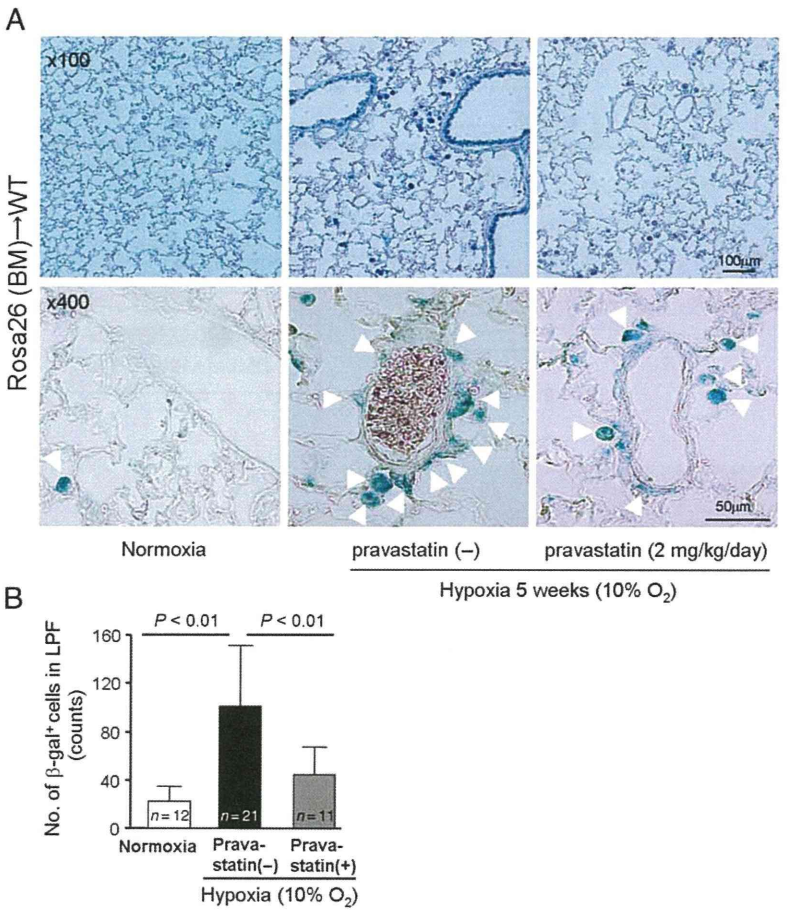

Figure 3 Bone marrow (BM)-derived cells in the adventitia of pulmonary arteries of chimeric mice. (*A*) X-gal staining showed that β-galactosidase (β-gal)$^+$ BM-derived cells were increased by hypoxia and accumulated to the pulmonary artery adventitia (arrowheads) in hypoxic mice, while the number of BM-derived cells was reduced by pravastatin (arrowheads). (*B*) The number of β-gal$^+$ cells was significantly increased in the hypoxic lung, which was significantly suppressed by pravastatin. Results are expressed as means ± SD. Normoxia, normoxic mice; hypoxia, mice exposed to 5 weeks of hypoxia (10% O_2) with or without pravastatin (2 mg/kg/day). LPF, low-power field (×200).

progenitors and have a potential to migrate and incorporate into the media and contribute to medial thickening.[26,36] These reports and our present findings suggest the existence of circulating progenitor cells for α-SMA$^+$ cells and their crucial roles in the pathogenesis of pulmonary vascular remodelling in hypoxia-induced PAH.

4.2 Statins and pulmonary arterial hypertension

It has been recently demonstrated that statins induce apoptosis of neointimal smooth muscle cells[37] and ameliorate monocrotaline-induced PAH in rats.[12] Statins have also been shown to prevent pulmonary artery muscularization and progression of PAH by protecting expression and activity of endothelial nitric oxide synthase (eNOS) at post-transcriptional level in hypoxia-induced PAH in rats.[14,38] Although the mechanisms underlying the beneficial effects of statins on PAH are not fully elucidated, these studies suggest that statins may improve endothelial function, at least in part, through NO-dependent mechanisms.[12,14,38] On the other hand, it also has been shown that statins mobilize progenitors and activate their function through up-regulation of eNOS.[15,16,18] However, in the present study, the number of circulating progenitors assessed by FACS analysis was significantly reduced by pravastatin in hypoxic mice. Therefore, the effect of statins on mobilization of progenitors under hypoxic conditions may have a complex mechanism (*Figure 6*).

Recently, it has been shown that SDF-1 plays a crucial role to mobilize and recruit BM-derived CXCR4$^+$ proangiogenic cells to the ischaemic tissue.[31] Moreover, it has been shown that the activated perivascular myofibroblasts produce abundant SDF-1, which plays a crucial role in the recruitment of BM-derived cells to the perivascular area.[32] Again, recruited CXCR4$^+$ cells secret angiogenic cytokines and promote the proliferation and differentiation of resident cells in vascular walls.[31,32] In the present study, we demonstrated that pravastatin significantly reduced the plasma levels of SDF-1, which could explain, at least in part, for the reduced mobilization of progenitors by statin. Pravastatin also suppressed the expression of ICAM-1 that is important for homing of BM-derived cells to the ischaemic tissue.[33] Taken together, we were able to demonstrate that pravastatin significantly reduces mobilization and homing of