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

Kimio Satoh, MD, PhD; Tetsuya Matoba, MD, PhD; Jun Suzuki, MD, PhD; Michael R. O’Dell, BS; Patrizia Nigro, PhD; Zhaqiang Cui, PhD; Amy Mohan, BS; Shi Pan, PhD; Lingli Li, MD, PhD; Zheng-Gen Jin, PhD; Chen Yan, PhD; Jun-ichi Abe, MD, PhD; Bradford C. Berk, MD, PhD

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, H2O2, O2−, and · OH), including NADPH oxidases, xanthine oxidase, the mitochondrial respiratory chain, lipooxygenases, 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 secreted oxidative stress–induced factors.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 cells14,15 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...
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 LacZlox−/−-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. 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. We replaced enhanced green fluorescent protein with full-length WT mouse CyPA carrying a Flag tag to make the LacZlox−/−-Flag-CyPA construct. Embryonic stem cells transfected by electroporation with linearized LacZlox−/−-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 germ-line transmission were identified by polypeptide chain reaction of DNA harvested from tail sniplets of weaned offspring. We obtained 9 germine 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 LacZlox−/−-CyPA transgenic mouse and SM22α-Cre mouse (C57BL/6J background) 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 LacZlox−/−-CyPA mice to SM22α-Cre mice 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. 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). 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. The carotid arteries were harvested, fixed for 24 hours, and embedded in paraffin, and cross sections (5 μm) were prepared. 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. 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 mouse (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% CO2/95% air as described previously. 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 L83583 to generate intracellular ROS 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).

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, and seeded (50,000 per 105 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% CO2 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.
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. 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. Because CyPA induces VCAM-1 in human umbilical...
vein ECs\textsuperscript{13} and is chemotactic for inflammatory cells,\textsuperscript{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\textsuperscript{−/−} mice (Figure 3E). In contrast, expression of VCAM-1 was much lower in ligated carotids of CyPA\textsuperscript{−/−} mice than in WT mice (Figure 3C, 3D, and 3E). The number of CD45\textsuperscript{+} inflammatory cells did not differ in sham arteries from WT versus CyPA\textsuperscript{−/−} mice (Figure 3G and 3I). There was a significant increase in CD45\textsuperscript{+} cells in ligated arteries of WT mice (Figure 3F and 3G), whereas there was only a small change in CyPA\textsuperscript{−/−} mice (Figure 3H and 3I). Quantification of CD45\textsuperscript{+} cells per intimal or medial area showed a 2-fold greater number in WT than in CyPA\textsuperscript{−/−} 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\textsuperscript{lox}\textsuperscript{−}-CyPA construct (Data Supplement Figure I) was prepared with the pZ/EG vector, and LacZ\textsuperscript{lox}\textsuperscript{−}-CyPA mice were crossed with SM22\textsuperscript{α}-Cre mice to achieve VSMC-specific expression. The vessels in Figure 4A through 4C are from control mice (LacZ\textsuperscript{lox}\textsuperscript{−}-CyPA\textsuperscript{−/-}/SM22\textsuperscript{α}-Cre\textsuperscript{−/-}) in which the transgene expressed is LacZ, as shown by the blue\textsuperscript{-}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 SM22\textsuperscript{α}-Cre to express FLAG-CyPA (SM22\textsuperscript{α}-Cre\textsuperscript{−/-}/LacZ\textsuperscript{lox}\textsuperscript{−}-CyPA). Specifically, there was no LacZ expressed, as shown by \textsuperscript{-}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 SM22\textsuperscript{α}-Cre was highly efficient (>90% of cells expressed FLAG, and <10% expressed...
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/H11002/H11002, 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 (LacZflox-CyPA+/H11001/SM22α/Cre−) and control (LacZflox-CyPA+/H11001/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,
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 increased compared with WT-CM (Figure 8E) and was dramatically greater than proliferation induced by KO-CM. These results indicate that the level of CyPA expression has powerful effects on VSMC proliferation.
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 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.
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 by Ki67 staining was significantly increased in ligated VSMC-Tg carotids compared with control carotids.

**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.

**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.
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.

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% CO2 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.
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. 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, 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 al32 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.

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Disclosures

None.

References


**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.
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