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.

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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

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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.

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ings, we demonstrated that AngII induces ROS and MMP activation via a CyPA-dependent pathway, a novel mechanism for induction of AAA formation by AngII.

Our data suggest that extracellular CyPA and its signaling pathways are novel targets for AAA therapy and, potentially, other cardiovascular diseases associated with inflammation. In addition, extracellular CyPA induces ROS production in VSMCs, which is consistent with our previous report that extracellular CyPA stimulates at least 3 signaling pathways (ERK1/2, Akt and JAK) in VSMCs,[36] which has been shown to be important for ROS production.[6,46] All these data are proof-of-concept that CyPA plays a crucial role in VSMCs through ROS generation. AngII induces the generation of ROS and promotes the secretion of CyPA. ROS-induced CyPA secretion augments ROS production synergistically (**Figure 1**). Subsequently, secreted CyPA, acting as a proinflammatory cytokine, synergistically augments AngII-mediated ROS production, contributing to the onset of vascular inflammatory cell migration and AAA formation (**Figure 3**).[62]

CyPA as a Potential Atherogenic Cytokine

Numerous basic and clinical studies have clearly identified that ROS have a major role in endothelial damage and the development of atherosclerosis.[73–75] However, we still do not have a strong therapeutic strategy for the clinical benefits of antioxidant administration. One potential reason for this could be the crucial role of ROS (especially H_2O_2) at very low concentration in intracellular signaling pathways that are also important for vascular functions.[30,31,76,77] CyPA (both intracellular and extracellular) contributes to atherosclerosis by promoting EC apoptosis and EC expression of leukocyte adhesion molecules, stimulating inflammatory cell migration, enhancing ROS production, increasing proliferation of macrophages and VSMCs, and increasing proinflammatory signal transduction in VSMCs.[78,79] In the context of atherosclerosis, CyPA can be regarded as a proinflammatory and proatherogenic molecule. CyPA is highly expressed at sites of unstable atherosclerotic plaques, especially those associated with macrophages and foam cells. However, CyPA expression and its regulatory molecular mechanisms during the process of plaque destabilization remain elusive and further research into the role of CyPA in the progression of atherosclerosis is needed to identify potential CyPA-related therapeutic targets.

CyPA as a Potential Promoter of Cardiac Hypertrophy

AngII plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy.[80] Therefore, understanding the molecular mechanisms responsible for AngII-mediated myocardial pathophysiology is critical to the development of new therapies for cardiac dysfunction.[81] One important mechanism now recognized as involved in AngII-induced cardiac hypertrophy is ROS production,[82,83] but the precise mechanism by which ROS cause hypertrophy remains unknown.[84] Our recent study provides strong mechanistic evidence of synergy between CyPA and AngII to increase ROS generation.[45] Because ROS stimulate myocardial hypertrophy, matrix remodeling, and cellular dysfunction,[85] CyPA will potentially enhance AngII-induced cardiac hypertrophy.

CyPA as a Potential Promoter of Hypoxia-Induced Pulmonary Arterial Hypertension (PAH)

PAH is associated with hypoxic exposure, enhanced ROS, and proliferation of VSMCs. Erythropoietin (Epo) has long been regarded as a hypoxia-induced hormone that acts exclusively in the proliferation and differentiation of erythroid progenitors. However, recent studies have demonstrated expression of the Epo receptor (EpoR) in the cardiovascular system,[86] and the therapeutic potential of Epo has been noted in a variety of disorders, including cerebral infarction, myocardial ischemia–reperfusion, and congestive heart failure. Recently, we demonstrated that the endogenous Epo/EpoR system plays an important protective role against the development of hypoxia-induced PAH.[87] For this purpose, we used $EpoR^{-/-}$-rescued mice that express EpoR only in the erythroid lineage, but not in th ecardiovascular system.[86] Moreover, we demonstrated the important role of the endogenous Epo/EpoR system in ischemia-induced regeneration and angiogenesis.[88]

Considering the role of CyPA in augmentation of ROS and VSMC proliferation and migration in vivo and in vitro, CyPA may potentially promote hypoxia-induced PAH. We have reported that Rho-kinase is activated in patients with PAH.[89] In addition, the secretion of CyPA is regulated by the Rho/Rho-kinase system.[38,45] Therefore, we tested the hypothesis that CyPA contributes to Rho-kinase activation and pulmonary vascular remodeling in PAH patients.[90] A key aspect of the study that deserves comment is the strong CyPA expression on αSMA-positive cells in the lungs of patients with idiopathic PAH. It is reported that bone marrow-derived αSMA-positive cells contribute to the development of PAH[91] and promote atherosclerotic plaque stability.[92] Additionally, we have reported that statins and a Rho-kinase inhibitor reduced the secretion of CyPA from VSMCs,[38,45] and demonstrated that pravastatin ameliorates hypoxia-induced PAH in mice.[93] Based on this, inhibition of CyPA secretion by statins[93] or Rho-kinase inhibitor[94,95] may contribute to the therapeutic effect of these drugs in PAH patients.

Conclusions

The identification of CyPA as a mediator of tissue damage associated with inflammation and oxidative stress provides insight into the mechanisms of several therapies. For example, the Rho-kinase inhibitor, Y27632, and simvastatin significantly reduced CyPA secretion from VSMCs. Rho-kinase is an important therapeutic target in cardiovascular disease[96] and Rho-kinase inhibition has been reported to reduce AngII-induced AAA formation,[97] atherosclerosis, and cardiac hypertrophy.[98] Moreover, AT1a receptor blockers and angiotensin-converting enzyme inhibitors have been shown to prevent cardiovascular diseases,[66,67,99] and reduced CyPA secretion may partially contribute to the therapeutic effect of these drugs on AAA, atherosclerosis, and cardiac hypertrophy.[45] EMMPRIN, a putative CyPA receptor, was identified as a tumor cell membrane protein that is expressed in VSMCs, activated by ROS and stimulates MMP production.[100] A recent study demonstrated ROS-dependent increases in EMMPRIN,[101] which may be activated by binding of extracellular CyPA.[102] Moreover, it has been demonstrated that EMMPRIN is strongly expressed in human AAA lesions[103] and in cardiomyocytes.[104] Therefore, it is logical to propose that agents that prevent CyPA binding to its receptors may have therapeutic potential (**Figure 1**). Because inflammation

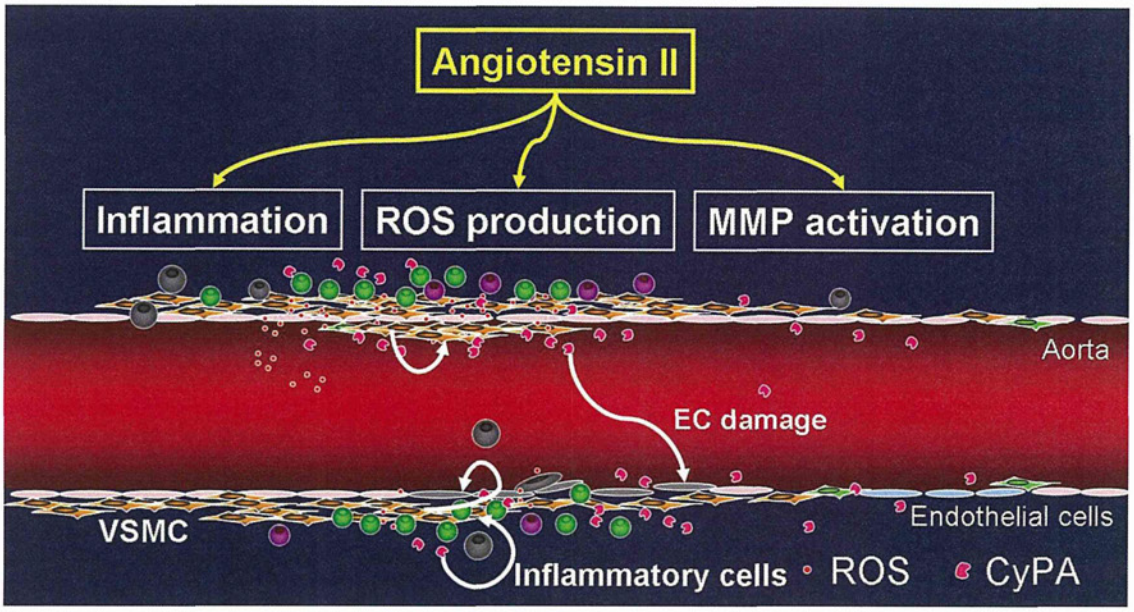

Figure 3. Proposed mechanisms for angiotensin II (AngII)-induced reactive oxygen species (ROS) production, cyclophilin A (CyPA) secretion, metalloproteinase (MMP) activation, and abdominal aorta aneurysm (AAA) formation. Secreted extracellular CyPA activates ERK1/2, Akt, and JAK in an autocrine/paracrine manner, which promotes proliferation and migration of vascular smooth muscle cells (VSMCs), MMP activation, and inflammatory cell migration.

the proliferation and migration of VSMCs via a paracrine manner. CyPA is expressed by all cell types participating in vascular pathology.[57] Additionally, extracellular CyPA has recently been found to induce interleukin (IL)-6 release in inflammatory cells.[58] Moreover, investigating CyPA function in monocyte/macrophage cell lines revealed that CyPA induces the expression of cytokines/chemokines such as tumor-necrosis factor α, monocyte chemotactic protein-1, IL-8, IL-1β and MMP-9 through a pathway that is dependent on nuclear factor-κB activation. In our carotid ligation model, we observed significant accumulation of $CD45^+$ inflammatory cells in the intima of ligated $CyPA^{-/-}$ carotids and the VSMC-specific overexpression of CyPA (VSMC-Tg) further enhanced the accumulation of inflammatory cells in the ligated carotids, supporting the important role of CyPA in mediating the recruitment of inflammatory cells (**Figure 2**).[56]

We propose that ROS generated locally by inflammatory cells cause VSMCs to release CyPA, which would then promote recruitment of inflammatory cells that release several proinflammatory cytokines. In addition, CyPA regulates the proteolytic activity necessary for the migration of inflammatory cells, through its activation of MMPs. Our study revealed 3 important pathologic consequences of CyPA activity in vivo. First, VSMC-derived secreted CyPA is mitogenic by virtue of its ability to promote VSMC proliferation. Second, secreted extracellular CyPA is proinflammatory because it stimulates the recruitment of inflammatory cells. Third, secreted CyPA promoted the pathological setting that exacerbated the generation of intracellular ROS in VSMC derived from mouse aorta (**Figure 2**).

CyPA Augments ROS Production and MMP Activation

As we have discussed, ROS stimulate secretion of CyPA from VSMCs, and that extracellular CyPA stimulates VSMC migration and proliferation (**Figure 1**).[35,36] Extracellular CyPA also stimulates EC adhesion molecule expression, and is a chemoattractant for inflammatory cells.[36,38,59] Furthermore, CyPA is upregulated in patients with rheumatoid arthritis and implicated because of its crucial role in MMP activation.[52] AngII infusion into $ApoE^{-/-}$ mice for 4 weeks promotes AAA formation.[60,61] In animal models of AAA, genetic and pharmacological inhibition of both ROS production[62,63] and MMPs[64,65] suppressed development of aneurysms. In that animal model, the AngII type 1 (AT_1) receptor in the vascular wall, but not in inflammatory cells, is required for the initiation of AngII-induced AAAs.[66] Furthermore, treatment with an AT_1 receptor blocker significantly suppressed aneurysm formation in $ApoE^{-/-}$ mice.[67] Therefore, we hypothesize that VSMC-derived CyPA augments AngII-induced ROS production, MMP activation, and inflammatory cell recruitment into the aortic VSMCs, contributing to AAA formation and progression.

CyPA Promotes AAA Formation and Aortic Rupture

In the cardiovascular system, AAA formation results from chronic inflammation of the aortic wall, associated with decreased medial VSMCs, and progressive destruction of structural components, particularly the elastic lamina.[68] Key mechanisms include VSMC senescence,[69] oxidative stress,[6,46] increased local production of proinflammatory cytokines[70] and increased activities of MMPs that degrade extracellular matrix.[71,72] As expected, AAA formation in the AngII-induced $ApoE^{-/-}$ model was completely prevented against a $CyPA^{-/-}$ background.[45] We also demonstrated that CyPA is highly expressed in the aorta of patients with AAA, and colocalizes with active forms of MMPs. Based on these find-

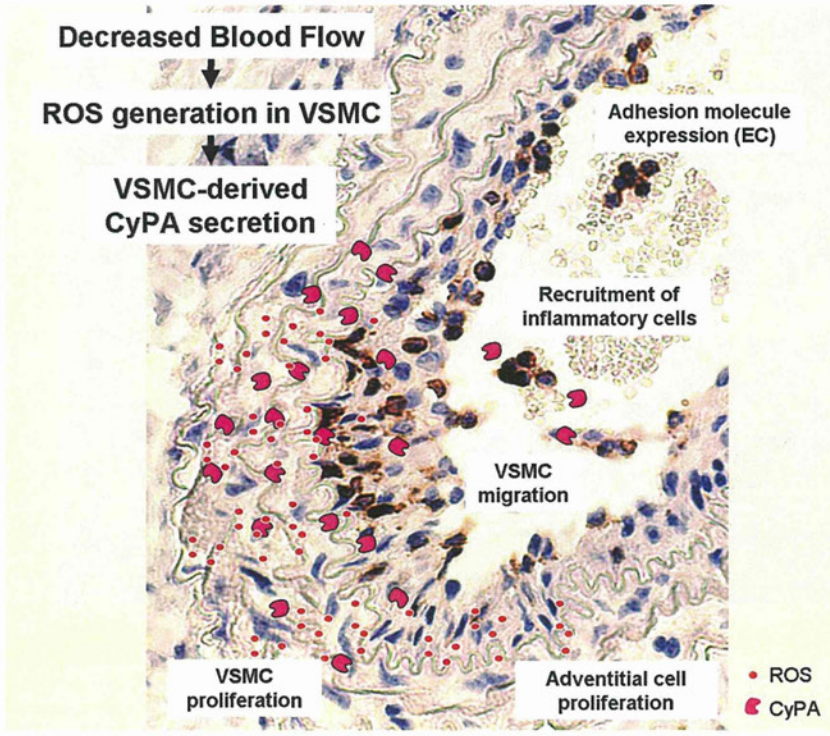

Figure 2. Cyclophilin A (CyPA) is a novel growth factor that mediates growth of vascular smooth muscle cells (VSMC) under oxidative stress. Decreased blood flow increases the generation of reactive oxygen species (ROS), which induces secretion of CyPA from VSMC. Secreted CyPA VSMC *proliferation*, *endothelial cell* (EC) adhesion molecule expression, and inflammatory cell migration (CD45-positive cells), resulting in vascular remodeling.

that cyclophilins serve multiple intracellular and extracellular functions, surprisingly little is known regarding their effect on specific receptors. Several molecules have been proposed as potential extracellular receptors for CyPA, including extracellular matrix metalloproteinase inducer (EMMPRIN).[39,40]

Mechanism of CyPA Secretion

It has been revealed that several growth factors are secreted from VSMCs in response to various stimuli.[41] CyPA is secreted from VSMCs via a highly regulated pathway that involves vesicle transport and plasma membrane binding.[38] Rho GTPases, including RhoA, Cdc42, and Rac1, are key regulators in signaling pathways linked to actin cytoskeletal rearrangement.[42] The Rho GTPases plays a central role in vesicular trafficking pathways by controlling the organization of the actin cytoskeleton. It has been reported that active participation of Rho GTPases is required for secretion of CyPA. We have shown consistently that the expression of dominant-negative mutants of RhoA and Cdc42 inhibited ROS-induced CyPA secretion, suggesting that both RhoA- and Cdc42-dependent signaling events regulate CyPA secretion.[38] Myosin II is involved in secretory mechanisms as a motor for vesicle transport.[43] Rho-kinase, a downstream effector of RhoA, mediates myosin II activation via phosphorylation and inactivation of myosin II light chain phosphatase.[44] We also demonstrated that a Rho-kinase inhibitor reduced ROS-induced CyPA secretion.[38,45] These results suggest that myosin II-mediated vesicle transport is required for CyPA secretion from VSMCs. CyPA is transported to the plasma membrane and colocalizes with VAMP in response to ROS stimulation. Therefore, CyPA is secreted from VSMCs through a process requiring ROS production and vesicle formation.

CyPA Promotes Intimal Thickness In Vivo

Increases in ROS represent a pathogenic mechanism for vascular disease.[46,47] ROS have been implicated in the pathogenesis of neointima formation, in part by promoting VSMC growth,[29,34] as well as by stimulating proinflammatory events.[48–51] We demonstrated that extracellular CyPA stimulates proinflammatory signals in ECs, including expression of E-selectin and VCAM-1.[37] In addition to the effects on vascular cells, CyPA has been shown to be a direct chemoattractant for inflammatory cells[52,53] and to promote matrix metalloproteinases (MMPs) activation.[54,55] Therefore, CyPA is a key mediator that affects ECs, VSMCs and inflammatory cell functions in vivo.

To confirm the role of CyPA in vascular remodeling, we observed the time course and distribution of its expression in carotid arteries after ligation.[56] We found that CyPA expression dramatically increased over a time course that paralleled neointimal formation, suggesting an important role for CyPA in the cellular response to oxidative stress induced by vascular injury. In parallel with CyPA expression, carotid ligation induced phosphorylation of ERK1/2 in wild-type carotids, which was significantly less in CyPA$^{-/-}$ carotids, consistent with the reduced number of Ki67^{+} cells in ligated CyPA$^{-/-}$ carotids. The distribution of Ki67^{+} cells closely overlapped with the areas of highest CyPA expression, especially in rapidly proliferating neointimal cells in WT mice. Colocalization of CyPA, α-smooth muscle actin (αSMA), and Masson-Trichrome staining revealed that CyPA expression was especially elevated in VSMCs. To prove further the contribution of VSMC-derived CyPA to vascular remodeling, we prepared VSMC-specific CyPA transgenic mice (VSMC-Tg). The observation that VSMC-specific CyPA overexpression not only increased the medial area but also the intimal area suggests that VSMC-derived extracellular CyPA promotes

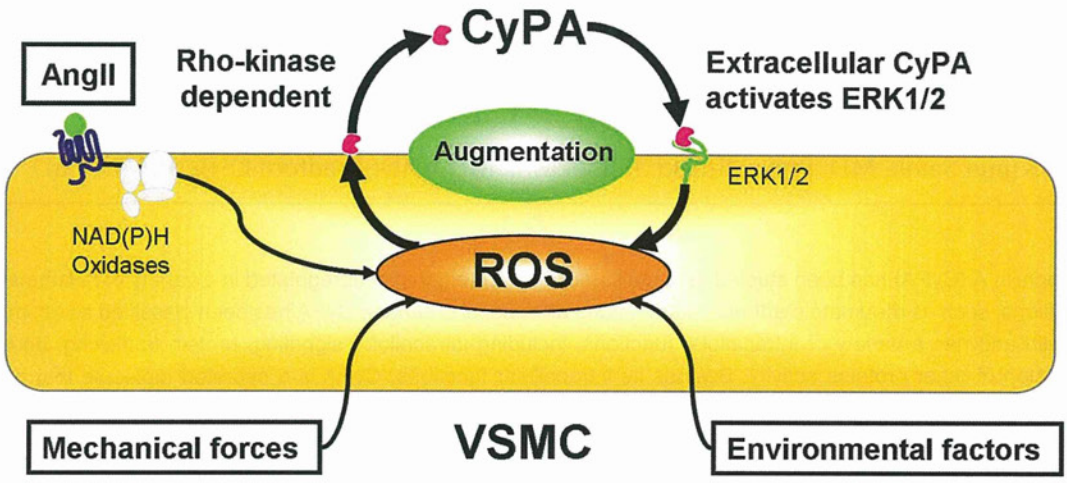

Figure 1. ROS-induced secretion of cyclophilin A (CyPA) synergistically augments ROS production. ROS inducers, such as angiotensin II (AngII), mechanical stress, and environmental factors, promote CyPA secretion from vascular smooth muscle cells (VSMC). Secreted CyPA activates ERK1/2 and promotes reactive oxygen species (ROS) production, thus augmenting it.

tion factor in protein folding and assembly. The first demonstration of this activity in vitro was delaying the maturation of collagen by blocking PPIase activity with CsA.[18] In addition to its role in protein folding, the PPIase activity of CyPA has recently been demonstrated to have other roles, including intracellular trafficking,[19] signal transduction, and transcription regulation.[20] Following the identification of CyPA, several other cyclophilins were cloned and characterized. Cyclophilin B (CyPB),[21] cyclophilin C (CyPC),[22] and cyclophilin D (CyPD)[23] were found to be less abundant and localized not only in the cytosol but also in membranes and subcellular organelles because of the presence of a hydrophobic N-terminal as well as C-terminal extensions. Human CyPB and murine CyPC are localized to the endoplasmic reticulum.[23] CyPD is localized to mitochondria and is an integral part of the mitochondrial permeability transition complex and plays a crucial role in apoptosis[24] and the pathogenesis of Alzheimer's disease.[25] A more detailed classification of the different cyclophilins has been reviewed recently.[13,26]

ROS in the Pathogenesis of Cardiovascular Disease

Production of intracellular ROS has been implicated in the pathogenesis of cardiovascular disease, in part by the promoting of VSMC proliferation.[27–29] Changes in vascular redox state are a common pathway involved in the pathogenesis of atherosclerosis, aortic aneurysms, and vascular restenosis after angioplasty. ROS target cellular biomolecules and cause severe damage, such as lipid peroxidation, protein oxidation/inactivation, and DNA damage/mutation. Although high levels of ROS might be hazardous to cells and their contents, controlled ROS levels (ie, physiological) are important in the regulation of cell functions and cell fate (proliferation/death). For example, H_2O_2 has also been implicated as important for EC function and vascular relaxation at very low concentrations.[30,31] In the vascular wall, ROS are generated by several mechanisms, including NADPH oxidases, xanthine oxidase, the mitochondrial respiratory chain, lipoxygenases and NO synthases.[32] Vascular ROS formation can be stimulated by mechanical stretch, pressure, shear stress, environmental factors such as hypoxia, and secreted factors such as angiotensin II (AngII).[33] We have demonstrated that ROS stimulate cultured VSMC proliferation and activate intracellular kinases such as ERK1/2 which is associated with cell growth.[29,34]

CyPA as a Secreted Oxidative Stress-Induced Factor (SOXF)

We found that activation of ERK1/2 by a ROS generator, napthoquinolinedione (LY83583), was biphasic (early and delayed activation). One explanation for the delayed ERK1/2 activation was the response to SOXF, which show autocrine/paracrine signals. In order to identify the presence of SOXF, we evaluated the ability of conditioned medium for ERK1/2 activation. The phosphorylation of ERK1/2 was significantly increased by conditioned medium from VSMCs treated with LY83583. Therefore, we analyzed the proteins released into the medium in response to LY83583 and finally found that CyPA is a major SOXF.[35] Furthermore, human recombinant CyPA stimulated ERK1/2 activity and DNA synthesis in VSMCs in a concentration-dependent manner.[36] Thus, we concluded that CyPA is a novel VSMC growth factor that contributes to the growth promoting activity of ROS in VSMCs.

Mechanism of CyPA-Induced VSMC Growth

Identification of the extracellular CyPA receptors is almost completely unexplored. We believe that further knowledge of the role played by extracellular CyPA receptors on vascular cell responses will help in designing therapeutics targeting inflammatory and cardiovascular diseases. In ECs, CyPA largely activates proinflammatory pathways, including increased expression of vascular cell adhesion molecule (VCAM)-1and E-selectin.[37] In VSMCs, ROS such as superoxide activate a pathway containing vesicles that results in secretion of CyPA.[38] Secreted extracellular CyPA stimulates ERK1/2, Akt and JAK in VSMCs, which contributes to ROS production again (**Figure 1**).[36] Despite the mounting evidence