Cyclophilin A Is Secreted by a Vesicular Pathway in Vascular Smooth Muscle Cells

Jun Suzuki, Zheng-Gen Jin,* David F. Meoli,* Tetsuya Matoba, Bradford C. Berk

Abstract—Reactive oxygen species (ROS) contribute to the pathogenesis of atherosclerosis in part by promoting vascular smooth muscle cell (VSMC) growth. Previously we demonstrated that cyclophilin A (CyPA) is a secreted oxidative stress–induced factor (SOXF) that promotes inflammation, VSMC growth, and endothelial cell apoptosis. However, the mechanisms that regulate CyPA secretion are unknown. In this study, we hypothesized that ROS-induced CyPA secretion from VSMC requires a highly regulated process of vesicle transport, docking, and fusion at the plasma membrane. Conditioned medium and plasma membrane sheets were prepared by exposing VSMC to 1 μmol/L LY83583, which generates intracellular superoxide. A vesicular transport mechanism was confirmed by colocalization at the plasma membrane with vesicle-associated membrane protein (VAMP). CyPA transport to the plasma membrane and secretion were significantly increased by LY83583. Reduction of VAMP-2 expression by small interfering RNA inhibited LY83583-induced CyPA secretion. Pretreatment with 3 μmol/L cytochalasin D, an actin depolymerizing agent, abrogated CyPA secretion. Infection with dominant-negative RhoA and Cdc42 adenovirus inhibited CyPA secretion by 72% and 63%, respectively, whereas dominant-negative Rac1 had a small effect (11%). Pretreatment with the Rho kinase inhibitor Y27632 (3 to 30 μmol/L) and myosin II inhibitor blebbistatin (1 to 10 μmol/L) inhibited CyPA secretion in a dose-dependent manner. Simvastatin (3 to 30 μmol/L) also dose-dependently inhibited LY83583-induced CyPA secretion likely via decreased isoprenylation of small GTPases. Our findings define a novel VSMC vesicular secretory pathway for CyPA that involves actin remodeling and myosin II activation via RhoA-, Cdc42-, and Rho kinase–dependent signaling events. (Circ Res. 2006;98:0-0.)

Key Words: cyclophilin A ■ reactive oxygen species ■ smooth muscle cells ■ atherosclerosis

Vascular smooth muscle cells (VSMC) respond to alterations in blood pressure and tissue demands by diverse growth responses that include proliferation, hypertrophy, and apoptosis. Abnormal VSMC growth contributes to the pathogenesis of atherosclerosis and restenosis. Increasing evidence indicates that autocrine and paracrine growth factors modulate VSMC growth in part by mediating secretion of growth factors,1 such as epiregulin,2 platelet-derived growth factor,3,4 and transforming growth factor-β.5 Reactive oxygen species (ROS) have been shown to be key growth factors both by direct effects or protein synthesis and cell growth pathways6,7 and by stimulating autocrine/paracrine growth pathways.

ROS induce VSMC growth6 by activating kinases such as extracellular signal-regulated kinases (ERK) 1/27 and p38.8 In a previous study, we observed that stimulation of VSMC with the naphthoquinolinedione LY83583, an intracellular superoxide generator, resulted in a biphasic activation of ERK1/2: a rapid phase of ERK1/2 activation was observed at 5 to 10 minutes and late phase was observed at 2 hours.9 These results led us to explore the existence of specific mediators of ROS that participate in the late phase activation of ERK1/2 by autocrine/paracrine growth mechanisms. Recently, we demonstrated that cyclophilin A (CyPA) is a secreted oxidative stress–induced factor (SOXF) that stimulates ERK1/2 and promotes VSMC growth and endothelial cell apoptosis.10,11 CyPA is a ubiquitously distributed and abundantly expressed intracellular protein that has peptidyl-prolyl cis-trans isomerase activity.12 CyPA has multiple intracellular functions, including roles as immunophilins that interact with calcineurin, as components of a caveolin–cholesterol–cyclophilin complex, and as components of the cell cycle.13 Evolving data point to novel extracellular roles of CyPA in inflammatory diseases such as rheumatoid arthritis14 and atherosclerosis.10,11 Extracellular CyPA stimulates IκB phosphorylation and nuclear factor κB activation and induces expression of adhesion molecules including E-selectin and vascular cell adhesion molecule-1.11 These results suggest that secreted CyPA acts as an inflammatory mediator that may be involved in the pathogenesis of atherosclerosis. However, the mechanisms that regulate CyPA secretion are unknown.
In this study, we hypothesized that ROS-induced CyPA secretion requires a highly regulated process of vesicle transport, docking and fusion at the plasma membrane. We show that Rho GTPase signaling, actin remodeling, and myosin II activation are involved in CyPA secretion. Furthermore, we show that 3 hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors dramatically decrease ROS-induced CyPA secretion by VSMC.

### Materials and Methods

#### Reagents and Chemicals

LY83583 and rabbit VAMP-2 antibody were from Sigma (St Louis, Mo); jasplakinolide, cytochalasin D, Y27632, blebbistatin, and simvastatin were from Calbiochem (La Jolla, Calif); rabbit CyPA polyclonal antibody was from BIOMOL Research Laboratories Inc (Plymouth Meeting, Pa); and goat vesicle-associated membrane protein (VAMP)-1/2 polyclonal and mouse c-myc monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif).

#### Cell Culture and Treatments

VSMC were isolated from 200 to 250 g male Harlan Sprague–Dawley rats and maintained in DMEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO2/95% air, as described previously.15 Passages 5 to 14 VSMC at 70% to 80% confluence were growth arrested by incubation in serum-free DMEM for 24 hours and stimulated by 1 μmol/L LY83583 or DMEM for 2 hours. For the inhibitor studies, cells were pretreated with 0.3 to 3 μmol/L jasplakinolide, 0.3 to 3 μmol/L cytochalasin D, 3 to 30 μmol/L Y27632, 1 to 10 μmol/L blebbistatin, or 3 to 30 μmol/L simvastatin for 30 minutes.

#### Preparation of Conditioned Medium

Conditioned medium from LY83583-stimulated cells or control medium from DMEM-incubated cells was collected and centrifuged for 10 minutes at 800g to remove cell debris. The medium was concentrated 100-fold by using a Centricron Plus-20 filter (Millipore Corporation, Bedford, Mass) to yield concentrated conditioned medium.

#### Plasma Membrane Sheet Assay

Preparation of plasma membrane sheets and immunofluorescence were performed as described previously.16 A detailed description of plasma membrane sheet assay is available in the online data supplement at http://circres.ahajournals.org.

#### Subcellular Fractionation

CyPA-containing vesicles were separated as described previously.17,18 (For details, see the online data supplement.)

#### RNA Interference

VSMC were treated with small interfering RNA (siRNA) against rat VAMP-2 coding region. The target sequences were 5'-AACAAAGTTGACGCGACGTTCAAG-3' for rat VAMP-2 and 5'-AATTCCTCCAGACTGTACGTTCA-3' for control nonsilencing siRNA (QIAGEN-Xeragon). VSMC at 50% confluence were transfected with 40 nmol/L of siRNA using Lipofectamine 2000 (Invitrogen) for 4 hours. Cells were harvested 48 hours later and used for experiments.

#### Adenovirus Transfection

Cells were infected with dominant-negative c-myc tagged N19RhoA, N17Cdc-42, and N17Rac1 mutant–expressing adenoviruses19 at a multiplicity of infection of 50. β-Galactosidase (LacZ)–expressing adenovirus (50 multiplicity of infection) was used as a control.

### Western Blot Analysis

The membrane was blocked for 2 hours at room temperature with 5% milk. The blot was incubated with the primary antibody (1:1000) overnight at 4°C, followed by incubation for 1 hour with horseradish peroxidase–conjugated secondary antibody. Immunoreactive bands were visualized using ECL (Amersham Pharmacia Biotech). Analysis of autoradiograms was performed by scanning densitometry and processing with NIH image analysis software.

### Statistical Analysis

All immunofluorescent images and immunoblots are representative of at least three to 4 experiments. Quantitative data are presented as mean±SE. Differences were analyzed with 1-way ANOVA and post hoc analysis was performed using Bonferroni–Dunn. P<0.05 was accepted as a significant difference.

### Results

#### Oxidative Stress Induces Secretion of Cyclophilin A From Vascular Smooth Muscle Cells

We have previously demonstrated that LY83583, an intracellular superoxide generator, induces secretion of CyPA from VSMC.10 We first examined the time course of CyPA secretion after LY83583 exposure by Western blot analysis of conditioned medium. CyPA secretion was observed following 30 minutes of exposure to LY83583 (1 μmol/L) with abundant secretion at 120 minutes, whereas the CyPA protein level did not change in total cell lysates (Figure 1A).

We postulated that CyPA is secreted through a process requiring vesicle formation. To examine the plasma membrane colocalization of CyPA and vesicular proteins, resulting from LY83583-induced transport of secreted CyPA vesicles, we double-stained isolated plasma membrane sheets16 with CyPA and VAMP antibodies. Under basal conditions, there was no CyPA immunofluorescence in the plasma membrane (Figure 1B). In contrast, LY83583 stimulation significantly increased CyPA immunofluorescence that was maximal at 1 hour (Figure 1B and 1C). When the plasma membrane sheets were stained for the scaffolding protein 14-3-3 as a negative control, no obvious expression was observed (data not shown). The colocalization of CyPA with VAMP in the plasma membrane suggests that LY83583 induces transport of CyPA vesicles to the plasma membrane (Figure 1D). To confirm further if CyPA is translocated from an intracellular compartment to plasma membrane associated vesicles in response to LY83583 stimulation, VSMC lysates were separated on a sucrose density gradient and CyPA and VAMP-2 detected by Western blot analysis. LY83583 exposure increased the level of CyPA in VAMP-2–enriched vesicle fractions (lanes 11 to 12) from 8.4% of total CyPA in controls to 24.0% in LY83583-exposed VSMC (supplemental Figure I). VAMP-2 has been shown to play a key role in secretion of synaptic vesicles.20 Thus we next examined the effect of VAMP-2 gene knockdown on CyPA secretion. Compared with control siRNA, VAMP-2 siRNA significantly inhibited LY83583-induced CyPA secretion (Figure 2). VAMP-2 siRNA had no effect on ERK1/2 activation by LY83583 stimulation for 10 minutes (data not shown). These results suggest that VAMP-2 expression and vesicle formation are required for CyPA secretion in VSMC.
Actin Remodeling Is Required for CyPA Secretion

Remodeling of the actin cytoskeleton is involved in intracellular trafficking mechanisms and plays a critical role in regulated secretion.21 Dynamic actin disassembly and reassembly are necessary for regulated membrane docking and fusion events.22,23 To determine whether actin polymerization and depolymerization are involved in CyPA secretion, VSMC were pretreated with the actin polymerizing agent jasplakinolide (0.3 to 3 μmol/L) or the depolymerizing agent cytochalasin D (0.3 to 3 μmol/L) for 30 minutes before exposure to 1 μmol/L LY83583. Both jasplakinolide and cytochalasin D inhibited LY83583-induced CyPA secretion in a dose-dependent manner (Figure 3).

Rho GTPases Regulate CyPA Secretion

Rho GTPases including RhoA, Cdc42, and Rac1 are well known for their effects on actin cytoskeleton remodeling. To assess the roles of Rho GTPases in CyPA secretion, VSMC were infected with recombinant adenoviruses that express dominant-negative mutants N19RhoA, N17Cdc42, and N17Rac1. Infection with N17Rac1 had only a modest inhibitory effect on CyPA secretion (11%) (Figure 4A and 4B). In contrast, infection with N19RhoA and N17Cdc42 inhibited CyPA secretion to a large extent compared with control LacZ-expressing adenovirus infected cells (72% and 63%, respectively) (Figure 4A and 4B). Infection with both N19RhoA and N17Cdc42 had no additive inhibitory effect on CyPA secretion (supplemental Figure II). Infection with DN19RhoA and N17Cdc42 also inhibited LY83583-induced transport of CyPA to the plasma membrane (Figure 4C). These results indicate that RhoA and Cdc42 but not Rac1 are required for LY83583-induced CyPA secretion.

Rho Kinase and Myosin II Activity Are Required for CyPA Secretion

Rho kinase, a downstream effector of Rho, induces myosin II light chain phosphorylation via inactivation of myosin II light chain phosphatase.24 Phosphorylation of myosin II light chain has been reported to facilitate transport of secretory proteins.25,26 To investigate the roles of Rho kinase and myosin II activity in CyPA secretion, VSMC were pretreated with the Rho kinase inhibitor Y27632 (3 to 30 μmol/L) or the myosin II inhibitor blebbistatin (1 to 10 μmol/L) before exposure to 1 μmol/L LY83583. Both Y27632 and blebbistatin inhibited CyPA secretion in a dose-dependent manner (Figures 5A and 6A, respectively). Moreover, pretreatment with 30 μmol/L Y27632 and 10 μmol/L blebbistatin abolished LY83583-induced transport of CyPA to the plasma membrane (Figures 5B and 6B, respectively). These results suggest that CyPA secretion is not passive but requires Rho kinase and myosin II activity for secretion.

Simvastatin Inhibits CyPA Secretion

Protein isoprenylation is essential for the function of Rho GTPases. HMG-CoA reductase inhibitors such as statins...
prevent isoprenylation and inhibit intracellular signaling mediated by Rho GTPases. To examine the effect of statins on CyPA secretion, VSMC were pretreated with simvastatin (3 to 30 μmol/L) before exposure to 1 μmol/L LY83583 for 2 hours. Conditioned medium (CM) and total cell lysate (TCL) were immunoblotted with antibody to CyPA. The results were quantified by densitometry of autoradiograms. B, Growth-arrested VSMC were pretreated with jasplakinolide for 30 minutes before exposure to 1 μmol/L LY83583 for 2 hours. CM and TCL were immunoblotted with antibody to CyPA. The results were quantified by densitometry of autoradiograms. Values are mean ± SE from 3 independent experiments. *P<0.05 compared with LY83583 only.

Discussion
The major findings in this study are that CyPA is secreted from VSMC via a highly regulated pathway that involves vesicle transport and plasma membrane binding (Figure 8). We showed that signaling pathways involving RhoA and Cdc42 for myosin II activation and actin remodeling play a crucial role in ROS-induced CyPA secretion. We also found that statins inhibited CyPA secretion. To our knowledge, these findings are the first to demonstrate the mechanisms for CyPA secretion from VSMC in response to ROS.

Autocrine and paracrine growth mechanisms are important in VSMC growth. Indeed, several growth factors are secreted from VSMC in response to various stimuli. However, the intracellular trafficking mechanisms that regulate secretion of these growth factors are not well understood. The best studied secretory pathway in VSMC involves tissue factor, the initiator of the clotting cascade. Tissue factor is released in microparticles that bud from the VSMC plasma membrane. We harvested medium from LY83583-exposed VSMC and performed high-speed centrifugation (100 000 g/1 hour) onto a 60% sucrose cushion. Although virtually all of the tissue factor activity (95%) has been shown to be precipitated by this technique, we did not find CyPA in microparticles supporting the vesicle pathway described here. This novel secretory pathway for CyPA involves Rho GTPase-myosin II activation and actin remodeling regulated vesicle transport, docking, and fusion process.

Soluble N-ethylmaleimide–sensitive factor attachment protein (SNAP) receptors (SNAREs) play a central role in the
control of membrane traffic events. VAMP/synaptobrevin, a member of SNAREs, together with other SNAREs including syntaxin and SNAP-25 families are required for secretion of synaptic vesicles. In this study, we showed that CyPA transported to the plasma membrane and colocalized with VAMP in response to ROS stimulation (Figure 1). In addition, gene silencing of VAMP-2 by siRNA significantly inhibited ROS-induced CyPA secretion (Figure 2). These results support the hypothesis that CyPA is secreted from VSMC through a process requiring vesicle formation.

It has become clear that actin remodeling participates in vesicular trafficking pathways. The involvement of actin polymerization in CyPA secretion was demonstrated by use of cytochalasin D, an actin depolymerizing agent (Figure 3A). Consistent with our results, inhibition of secretory vesicle fusion with the plasma membrane by actin depolymerizing agents has been reported. However, actin stabilizing agents such as phalloxin and jasplakinolide also inhibit vesicle docking with plasma membrane. Indeed, jasplakinolide dose-dependently inhibited CyPA secretion (Figure 2). These observations indicate that regulated disassembly and reorganization of actin cytoskeleton plays an important role in secretory-vesicle trafficking and fusion.

Rho GTPases including RhoA, Cdc42, and Rac1 are key regulators in signaling pathways linked to actin cytoskeletal rearrangement. The Rho GTPases are therefore thought to have a central role in vesicular trafficking pathways by controlling organization of the actin cytoskeleton. In support of this notion, it has been reported that active participation of Rho GTPases is required for secretion. In agreement with these observations, we showed that expression of dominant-negative mutants of RhoA and Cdc42 inhibited ROS-induced CyPA secretion (Figure 4), suggesting that both RhoA- and Cdc42-dependent signaling events regulate CyPA secretion. Although Rac1 controls the organization of the actin cytoskeleton, expression of dominant-negative Rac1 had only a modest effect on CyPA secretion. A possible explanation for this finding is that the role of Rac1 in secretion is cell specific.

Myosin II is involved in secretory mechanisms as a motor for vesicle transport. Regulation of myosin II activity involves multiple cooperative pathways. For example, Rho kinase, a downstream effector of RhoA, mediates myosin II activation via phosphorylation and inactivation of myosin II light chain phosphatase. Recently, the Cdc42 target myotonic dystrophy kinase-related Cdc42 binding kinase was reported to cooperate with Rho kinase in myosin II activation. Our data show that both Y27632, a specific Rho kinase inhibitor, and blebbistatin, an inhibitor of myosin II ATPase activity, inhibited ROS-induced CyPA secretion and transport to the plasma membrane (Figures 5 and 6). These results suggest that myosin II–mediated vesicle transport is required for CyPA secretion from VSMC.
Recent clinical trials show that statins reduce cardiovascular events, mortality, and progression of atherosclerosis. Some of these beneficial effects of statins appear to be independent of cholesterol lowering, such as inhibiting inflammation and improving endothelial function. Statins prevent isoprenylation of Rho GTPases, thus inhibiting their membrane translocation and activation. Inhibition of Rho GTPases may represent 1 of the cholesterol-independent vascular protective effects of statins. Accordingly, our findings that Rho GTPases play a positive role in secretion mechanisms led us to study the effect of statins on CyPA secretion. We found that simvastatin inhibited ROS-induced transport of CyPA to the plasma membrane and secretion into conditioned medium (Figure 7). These results are consistent with previous findings that statins prevent secretion in alveolar epithelial cells. Inhibition of ROS-induced CyPA secretion may represent 1 of the vascular protective effects of statins.

Acknowledgments

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Figure 7. Simvastatin inhibits LY83583-induced CyPA secretion. A, Growth-arrested VSMCs were pretreated with simvastatin for 30 minutes before exposure to 1 µmol/L LY83583 for 2 hours. Conditioned medium (CM) and total cell lysate (TCL) were immunoblotted with antibody to CyPA. The results were quantified by densitometry of autoradiograms. B, Growth-arrested VSMCs were pretreated with 30 µmol/L simvastatin for 30 minutes before exposure to 1 µmol/L LY83583 for 60 minutes. Plasma membrane sheets were prepared and then fixed and stained for CyPA. Values are mean±SE from 4 independent experiments.

Figure 8. Proposed mechanisms for LY83583-induced CyPA secretion in VSMC. ROS-induced CyPA secretion requires an active process including vesicle formation, vesicle transport to the plasma membrane (requiring myosin II), docking, and fusion (requiring actin cytoskeleton remodelling). Rho, Cdc42, and Rho kinase may play an important role in both myosin II activation and actin remodelling.


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Cyclophilin A is secreted by a vesicular pathway in vascular smooth muscle cells

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Materials and Methods

Plasma membrane sheet assay

Cells cultured on 60-mm dishes were rinsed once in ice-cold phosphate-buffered saline (PBS) and incubated with 0.55 mg/ml of poly-L-lysine in PBS for 60 seconds. The cells were then swollen in a hypotonic buffer (23 mM KCl, 10 mM Hepes, pH 7.5, 2 mM MgCl₂, 1 mM EGTA) by three rinses. The swollen cells were probe-sonicated for 5 seconds at 25 watts and 10% power output with an Ultrasonic homogenizer 4710 Series (Cole Parmer Instrument Co., Chicago IL) with a 5-mm microtip set 1 cm above the surface of the cell monolayer in 10 ml of sonication buffer (70 mM KCl, 30 mM Hepes, pH 7.5, 5 mM MgCl₂, 3 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The bound plasma membrane sheets were washed three times with sonication buffer. The sheets were fixed for 20 minutes in a solution containing 2% paraformaldehyde, 70 mM KCl, 30 mM HEPES, pH 7.5, 5 mM MgCl₂, and 3 mM EGTA. The sheets were then blocked in 10% donkey serum for 60 minutes. After fixing and blocking steps the sheets were incubated with 1:200 dilution of CyPA and VAMP antibodies for 1 hour followed by incubation with 1:1000 dilution of Alexa Fluor 546 anti-rabbit IgG (H+L) for red and Alexa Fluor 488 anti-goat IgG (H+L) for green (Molecular Probes, Inc., Eugene, OR) for 1 hour. The intensity of the fluorescence was analyzed and quantified by NIH image analysis software.

Subcellular fractionation

Cells cultured on 100-mm dishes were rinsed three times in ice-cold PBS and harvested. The cells were then pelleted at 1,000 x g for 5 minutes and homogenized by 30 strokes with a Dounce homogenizer and 3 x 10 seconds bursts with an Ultrasonic homogenizer 4710 Series (Cole Parmer Instrument Co., Chicago IL) in a hypotonic buffer (5 mM Hepes, pH 7.4, 1 mM EGTA, 1
μg/ml leupeptin and 2 μg/ml aprotinin). The cell debris and the nuclei were eliminated by centrifuging the homogenate for 10 minutes at 3,000 x g. The supernatant was loaded onto a continuous sucrose gradient (0.45-2 M, 8 ml) and centrifuged at 110,000 x g for 18 hours in a Surespin 630/17 rotor; 16 fractions of 0.5 ml each were collected starting at the top of the gradient and an equal volume of each fraction was analyzed by Western blotting.

**Figure legends**

**Figure S1.** LY83583 induces translocation of CyPA to vesicle fraction. Growth-arrested VSMC were exposed to 1 μmol/L LY83583 for 1 hour. Subcellular fractions were isolated by use of sucrose gradient separation. Fractions of equal volume containing 0.45-2 M sucrose were immunoblotted with antibodies to CyPA and VAMP-2.

**Figure S2.** Effect of simvastatin on LY83583-induced CyPA secretion in dominant-negative RhoA infected VSMC. VSMC were infected with recombinant adenoviruses that express dominant-negative mutants N19RhoA, N17Cdc42 and both N19RhoA and N17Cdc42. Cells were subsequently pretreated with simvastatin for 30 min prior to exposure to 1 μmol/L LY83583 for 2 hours. Conditioned medium (CM) and total cell lysate (TCL) were immunoblotted with antibody to CyPA and c-myc.
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Supplemental Figure S2

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