Cyclophilin A Is a Proinflammatory Cytokine that Activates Endothelial Cells

Zheng-Gen Jin, Andreea O. Lungu, Liang Xie, Meng Wang, Chelsea Wong, Bradford C. Berk

Objective—Cyclophilin A (CyPA) is an abundant intracellular protein that is considered to be the main target of the immunosuppressive drug cyclosporine A. We and others showed that CyPA is secreted from smooth muscle cells and macrophages in response to oxidative stress and lipopolysaccharide, suggesting a role for CyPA in inflammation. We therefore studied the proinflammatory effects of CyPA on vascular endothelium.

Methods and Results—Because atherosclerosis is an inflammatory disease, we studied expression of CyPA in atherosclerotic plaques from the ApoE−/− mouse. Using immunohistochemistry, we showed that CyPA was highly expressed in these plaques. Because endothelial cells (EC) are important mediators of inflammation, we next studied the ability of CyPA to activate EC. Human recombinant CyPA activated mitogen-activated protein kinases, including ERK1/2, JNK, and p38 in cultured human umbilical vein EC. CyPA also stimulated IκB-α phosphorylation and NF-κB activation, and induced expression of adhesion molecules including E-selectin and vascular cell adhesion molecule-1. Furthermore, the combination of CyPA and cycloheximide induced EC apoptosis similar to the proapoptotic effect of tumor necrosis factor-α.

Conclusions—Our data indicate that CyPA has proinflammatory effects on EC and may play an important role in the pathogenesis of inflammatory diseases, such as atherosclerosis. **(*Arterioscler Thromb Vasc Biol*. 2004;24:1186-1191.)**

Key Words: cyclophilin A ■ mitogen-activated protein kinases ■ adhesion molecules ■ endothelial cell ■ atherosclerosis

Increasing evidence suggests that reactive oxygen species (ROS; ie, H_2O_2, O_2^-, and ·OH) represent a common pathogenic mechanism for atherosclerosis.[1,2] A particularly important mechanism for ROS-mediated cardiovascular disease appears to be via stimulation of proinflammatory events.[3,4] ROS formation can be stimulated by mechanical stress, environmental factors, platelet-derived growth factor, angiotensin II, and low-density lipoproteins.[5–7] Because many risk factors for coronary artery disease such as hyperlipidemia, hypertension, diabetes, and smoking increase ROS production, it has been suggested that changes in vessel redox state are a common pathway in the pathogenesis of atherosclerosis.[1,8,9] Recent data suggest a role for specific mediators of ROS in the development of atherosclerosis. We have defined one such family of mediators that we term SOXF for secreted oxidative stress-induced factors that includes cyclophilin A (CyPA), cyclophilin B (CyPB), and heat shock protein 90 (HSP90).[10,11] Plasma cyclophilin levels have been shown to be greatly increased in patients with sepsis and rheumatoid arthritis consistent with a role in inflammation.[12,13]

CyPA is a ubiquitously distributed protein belonging to the immunophilin family[14] and is recognized as the host cell receptor for the potent immunosuppressive drug cyclosporine A.[15,16] CyPA has also been shown to possess peptidylprolyl cis-trans-isomerase activity and is thought to play an important role in protein folding.[14,17] Although CyPA was initially believed to exist solely as an intracellular protein, later studies have revealed that it can be secreted by cells in response to inflammatory stimuli.[18,19] Secreted CyPA is a potent chemoattractant for monocytes,[18] neutrophils,[18,19] eosinophils,[19] and T cells[20] in vitro. It also elicits an inflammatory response characterized by a rapid influx of neutrophils when injected in vivo.[19] Recently, we found that CyPA was secreted from rat aortic vascular smooth muscle cells (VSMC) in response to ROS, and from mouse fibroblasts transfected with *nox1* [a superoxide-generating homologue of the phagocyte NAD(P)H oxidase catalytic subunit].[11] We demonstrated that secreted CyPA stimulated ERK1/2 and DNA synthesis in VSMC.[11] CyPA expression was highly regulated as shown by a dramatic increase in balloon-injured rat carotid.[11]

In this study, we hypothesized that secreted CyPA has proinflammatory effects on human vascular endothelium. Here we show that CyPA is highly expressed in atherosclerotic plaques from ApoE−/− mice, and human recombinant

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CyPA stimulates endothelial activation, expression of adhesion molecules, and apoptosis.

Methods

Immunohistochemistry

Immunohistochemistry was performed as described previously.[11] Atherosclerotic plaques were obtained from ApoE−/− mice (Jackson Laboratory) fed a Western diet for 12 weeks.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) obtained from collagenase-digested umbilical veins were cultured in RPMI medium with 20% bovine calf serum (Hyclone), 90 μg/mL heparin (Sigma), and endothelial cell growth factor (50 μg/mL) prepared from bovine brain.[21] All cells were maintained at 37°C in 5% CO_2. Experiments were performed with cells of passage 4. Cells were stimulated with human recombinant CyPA (BIOMOL) or human recombinant tumor necrosis factor-α (TNFα) (Sigma) as indicated.

Immunoblot Analysis

Total cell lysates were harvested and protein samples were separated on tris-glycine gels by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech).[22] Membranes were blotted with the relevant antibodies (phospho-ERK 1/2, phospho-p38, and phospho-IκBα; Cell Signaling Technology). After rinsing, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and visualized by enhanced chemiluminescence. The membranes were reprobed with anti-ERK1/2 or anti-p38 antibodies for total ERK1/2 and p38. C-Jun NH2-terminal kinase (JNK) activity was measured with a commercially available kit based on phosphorylation of recombinant c-Jun (Cell Signaling Technology), as described previously.[21]

NF-κB Luciferase Assay

The NF-κB reporter gene system was from Stratagene (PathDetect Reporting Systems; La Jolla, Calif). Transfection of HUVECs was performed by DEAE-dextran as described.[23] Luciferase activity followed by *renilla* activity was measured using a plate reader. All data were normalized as relative luciferase light units/renalia unit.

Enzyme-Linked Immunosorbent Assay for Cell Adhesion Molecules on HUVECs

To detect the expression of adhesion molecules, enzyme-linked immunosorbent assay (ELISA) was performed as described.[24] HUVECs were grown to confluence in 96-well plates, then treated with stimuli for 6 hours, and cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) and blocked with 0.1% bovine serum albumin in PBS. Then cells were incubated with the primary antibodies (anti–E-selectin monoclonal antibody, clone P2H3, or anti–VCAM-1 monoclonal antibody, clone 4B9; Chemicon) and secondary horseradish peroxidase-conjugated antibodies. Finally, cells were incubated with 1 mg/mL of o-phenylenediamine (Sigma) in 0.1 mol/L sodium citrate, pH 4.5, with 0.01% H_2O_2 (v/v), and the reactions were stopped with 3N HCl. Absorbance at 490 nm was measured for each well using a plate reader.

HUVECs Viability Assay

HUVECs grown in 96-well plate were treated with CyPA or TNF-α with or without CHX for 24 hours at 37°C. The medium was removed and replaced with medium containing 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) and incubated at 37°C for 5 hours. The medium was aspirated, and the formazan product was solubilized with dimethyl sulfoxide. Absorbance at 590 nm was determined for each well.[10,11]

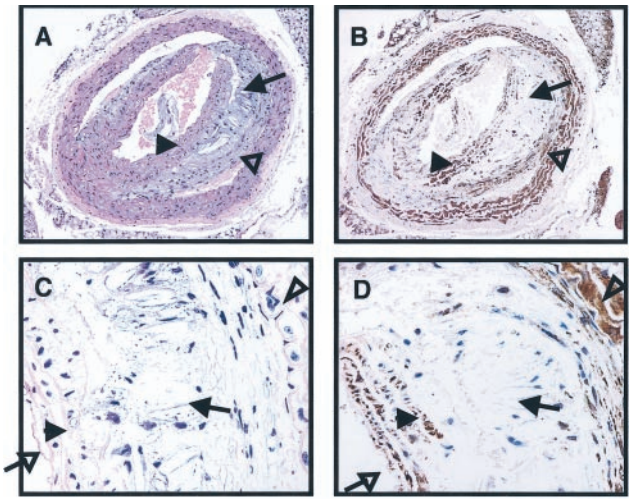

Figure 1. Immunostaining of CyPA in atherosclerotic plaques from apoE−/− mice. Sections of aortic sinus lesions of ApoE−/− mice after 12 weeks of Western diet were stained with H&E or polyclonal anti-CyPA antibodies. A and C, 10× and 40× H&E staining, respectively. B and D, 10× and 40× CyPA staining with anti-CyPA antibodies, respectively. Solid arrowhead indicates VSMC in media, solid arrow indicates cholesterol clefts, open arrowhead indicates extracellularly near the elastic lamina, and open arrow indicates endothelial cells. Results are representative of 4 vessels.

Endothelial Cell Apoptosis

Apoptotic cells were identified by morphological staining of nuclei as described before.[10,11] HUVECs were centrifuged (10 minutes, 700*g*), fixed in 3.7% formaldehyde, and stained with DAPI (4,6-diamidno-2-phenyindole; Sigma) for 20 minutes. Cells were photographed under a Nikon fluorescence microscope, and apoptotic cells were identified by their typical morphological appearance: chromatin condensation, nuclear fragmentation, or apoptotic bodies. Three hundred cells were counted by 2 independent blinded investigators, and the percentage of apoptotic cells per total number of cells was determined.

Statistical Analysis

Data are expressed as mean±SEM from at least 3 independent experiments or mean±SD of triplicate wells in a single experiment when indicated as such. Statistical analysis was performed with ANOVA followed by Student 2-tailed *t* test.

Results

CyPA Is Expressed in ApoE−/− Mouse Atherosclerotic Plaques

To demonstrate the role of CyPA in vivo, we showed previously that CyPA expression is increased in the rapidly proliferating neointimal cells within the balloon-injured artery of rats.[11] To extend these observations to an atherosclerotic model, the aortic root of ApoE−/− mice fed a Western diet was studied (Figure 1). H&E staining (Figure 1A and 1C) shows a large atherosclerotic plaque with cholesterol clefts (solid arrow) and VSMC proliferation (open arrow). Staining for CyPA with anti-CyPA antibodies (Figure 1B and 1D) shows specific expression in VSMC (solid arrowhead) and extracellularly near elastic lamina (open arrowhead), but not in the cholesterol clefts (solid arrow). There is some staining of CyPA in endothelial cells at vessel lumen (open arrow). No significant fluorescence was observed in the negative control

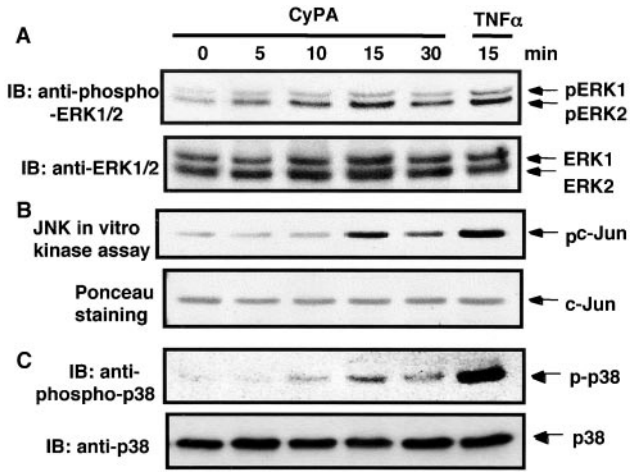

Figure 2. CyPA stimulates time-dependent activation of ERK1/2, JNK, and p38 in HUVECs. Growth arrested HUVECs were stimulated with 10 nM CyPA for the indicated times or 10 ng/mL TNFα for 15 minutes. Activity of ERK1/2 (A), JNK (B), and p38 (C) was measured as described in Methods. The membranes were reprobed with anti-ERK1/2 or anti-p38 antibodies for total ERK1/2 and p38. Blots are representative of 3 independent experiments.

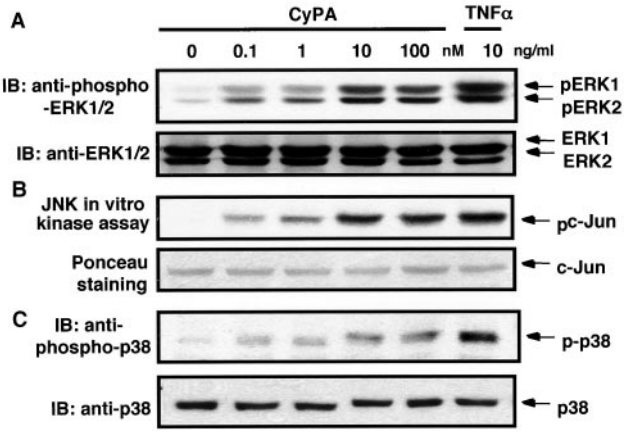

Figure 3. CyPA stimulates concentration-dependent activation of ERK1/2 (A), JNK (B), and p38 (C) in HUVECs. Growth-arrested HUVECs were stimulated with the indicated concentrations of CyPA for 15 minutes or 10 ng/mL TNFα for 15 minutes. Activity of ERK1/2, JNK, and p38 was measured as described in Methods. The membranes were reprobed with anti-ERK1/2 or anti-p38 antibodies for total ERK1/2 and p38. Blots are representative of 3 independent experiments.

of normal aortic vessels processed by omitting the primary anti-CyPA antibodies (data not shown).

CyPA Stimulates Activation of Mitogen-Activated Protein Kinases in Endothelial Cells

To determine whether human recombinant CyPA stimulates mitogen-activated protein kinases (MAPK) in endothelial cells (ECs), HUVECs were exposed to 10 nM CyPA for different times. As a positive control, cells were also exposed to 10 ng/mL TNFα for 15 minutes.[21] ERK1/2, JNK, and p38 activities were then analyzed by immunoblotting. ERK1/2 was activated most rapidly (start at 5 minutes and peak at 15 minutes) and to a similar extent as seen with TNFα (Figure 2A). JNK was activated within 15 minutes after stimulation by CyPA in HUVECs (Figure 2B). Peak JNK activation (4.5$\pm$0.6-fold increase) occurred at 15 minutes (Figure 2B). The p38 activation by CyPA was relatively weak ($\approx$3-fold at the peak of 15 minutes) compared with TNFα ($\approx$10-fold) (Figure 2C). The preparation of human recombinant CyPA used for these studies was highly purified as shown by silver stain analysis, which revealed that $>$95% of total protein migrated at a molecular weight of 18 kDa, consistent with CyPA.[11] Stimulation of MAPK by CyPA was not caused by the contamination of endotoxin because heat treatment (boiling for 20 minutes) of human recombinant CyPA abrogated its activity.[11]

We also determined the concentration dependence for MAPK activation by CyPA in HUVECs (Figure 3). ERK1/2 was activated by CyPA at 0.1 nM, and was maximal at 10 nM, with half-maximal effect at $\approx$1 nM (Figure 3A). CyPA also stimulated concentration-dependent increases in JNK and p38 activity (Figure 3B and 3C).

CyPA Stimulates of NF-κB Transcriptional Activity in HUVECs

To determine whether human recombinant CyPA stimulates activation of NF-κB in EC, HUVECs were exposed to 10 nM CyPA for different times or 10 ng/mL TNFα for 15 minutes. Immunoblotting studies were performed to determine phosphorylation of IκBα. Analysis of HUVECs extracts using phospho-specific IκBα antibodies showed that CyPA increased phosphorylation of IκBα with a peak at 15 minutes (Figure 4A). To further determine the effect of CyPA on NF-κB transcriptional activity, HUVECs were transfected

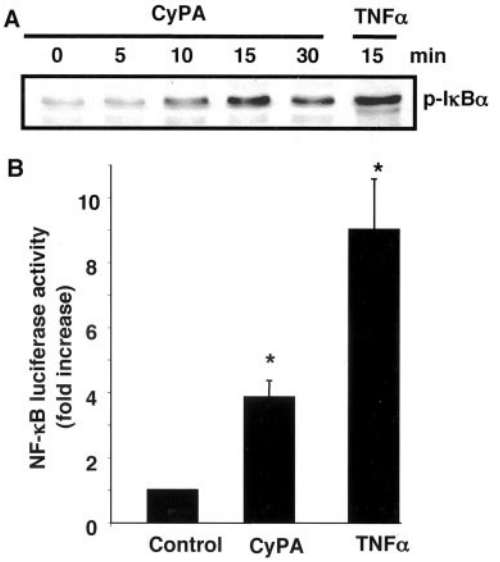

Figure 4. CyPA stimulates phosphorylation of IκBα (A) and activation of NF-κB (B) in HUVECs. A, Growth-arrested HUVECs were stimulated with 10 nM CyPA for the indicated times or 10 ng/mL TNFα for 15 minutes. Phosphorylation of IκBα was measured as described in Methods. Blots are representative of 3 independent experiments. B, HUVECs were transfected with NF-κB reporter gene luciferase and stimulated with 10 nM CyPA or 10 ng/mL TNFα for 6 hours. Induction of NF-κB transcriptional activity was measured as the luciferase activity in the experimental cells relative to those in controls. Data are represented as mean$\pm$SEM from 4 separate experiments. *$P<0.05$ versus control.

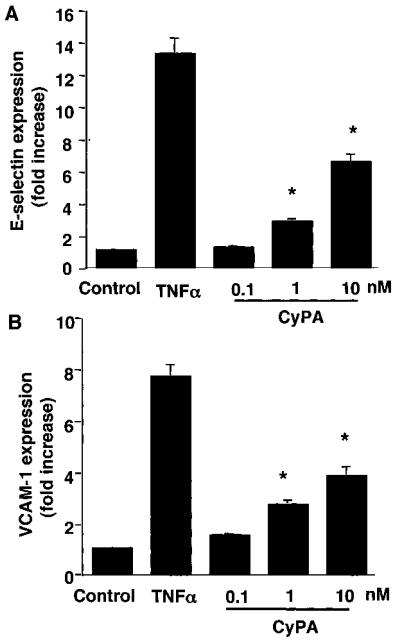

Figure 5. CyPA induces E-selectin and VCAM-1 expression in HUVECs. Growth-arrested HUVECs were stimulated with the indicated concentrations of CyPA or 10 ng/mL TNFα. After 6-hour incubation, HUVECs were fixed. E-selectin and VCAM-1 expression on cell surface were measured by ELISA as described in Methods. Results shown are the means±SD of triplicate wells in a single experiment and are representative of 3 separate experiments. *$P<0.05$ versus control.

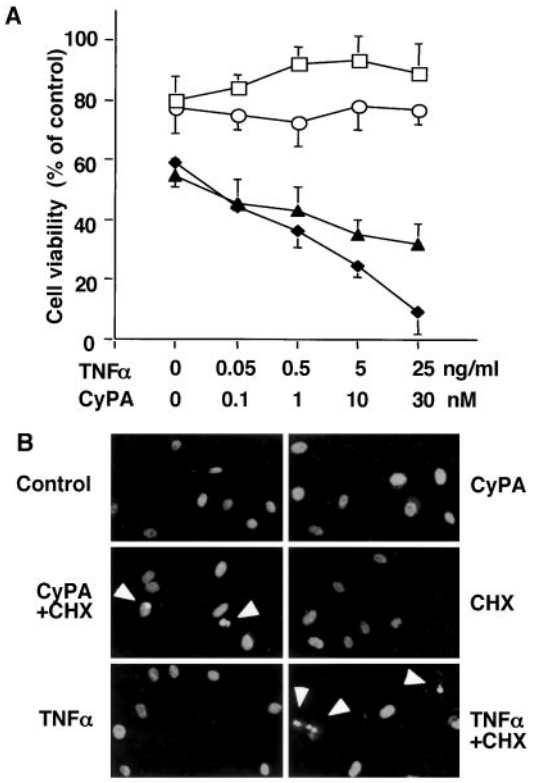

Figure 6. CyPA induces cell death in the presence of CHX. A, HUVECs were incubated with the indicated concentrations of CyPA or TNFα in absence or presence of 10 μg/mL CHX. An MTT assay was performed after 24-hour incubation. Percentage of viability was expressed as the proportion of untreated cells. Results shown are the means±SD of triplicate wells in a single experiment and are representative of 3 separate experiments. □ indicates TNFα alone; ♦, TNFα + CHX; ○, CyPA alone; ▲, CyPA + CHX. B, HUVECs were treated with 10 μg/mL CHX, 10 nM CyPA, 10 ng/mL TNFα, or indicated combinations. After 16 hours, HUVECs were fixed, and apoptotic cells were identified by morphological staining of nuclei described in Methods. Results shown are representative of 3 separate experiments (B).

with NF-κB luciferase construct and exposed to 10 nM CyPA or 10 ng/mL TNFα for 6 hours. CyPA significantly stimulated NF-κB luciferase activity (3.6±0.2-fold versus control) to ≈40% of that observed in response to TNFα (Figure 4B).

CyPA Induces E-Selectin and VCAM-1 Expression on HUVECs

To gain further insight into the functional significance of MAPK and NF-κB activation by CyPA, we determined whether CyPA induces expression of cell adhesion molecules. HUVECs were exposed to CyPA or TNFα for 6 hours, and expression of E-selectin and VCAM-1 was measured by ELISA. CyPA induced both E-selectin and VCAM-1 expression in a dose-dependent manner, to ≈50% of that induced by 10 ng/mL TNFα (Figure 5A and 5B).

CyPA Triggers Endothelial Cell Apoptosis

Previous studies have demonstrated that TNFα induced HUVECs apoptosis when protein synthesis was inhibited by cycloheximide (CHX) or actinomycin D.[25,26] To determine whether CyPA induces HUVECs apoptosis, HUVECs were treated for 24 hours with 10 nM CyPA or 10 ng/mL TNFα plus 5 μg/mL CHX. The cell viability was assayed by MTT. Both CyPA and TNFα induced cell death in the presence of CHX in a dose-dependent manner (Figure 6A). To show that the cell death was caused by apoptosis, cell nuclei were analyzed by fluorescence microscopy. There is no significant induction of apoptosis by the treatment of vehicle, CyPA, CHX, and TNFα, individually (Figure 6B). The combination of TNFα with CHX induced significant apoptosis of HUVECs to 36.2±6.1% compared with control (vehicle). Similar to TNFα, CyPA plus CHX induced cell apoptosis to 15.4±1.4% compared with control (Figure 6B), suggesting HUVECs survival after exposure to CyPA is dependent on synthesis of a cytoprotective protein(s).

Discussion

The major finding of this study is that CyPA activates EC in a proinflammatory manner. First, we found that CyPA was highly expressed in atherosclerotic plaques from ApoE−/− mice. We next demonstrated in HUVECs that CyPA activated MAPK and NF-κB, and it increased expression of adhesion molecules. Finally, we showed that CyPA induced EC apoptosis similar to the proapoptotic effect of TNFα. These results suggest a potential role of CyPA in the pathogenesis of atherosclerosis.

CyPA is a ubiquitously distributed protein belonging to the immunophilin family. Although CyPA is usually local-

ized within cells, its extracellular release has been shown to occur in macrophages activated by lipopolysaccharide and in VSMC in response to oxidative stress and vascular injury.[10,11,18] Previously, we found that CyPA was secreted from VSMC in response to O_2^- and H_2O_2, and from fibroblasts transfected with *nox1* [a superoxide-generating homologue of the phagocyte NAD(P)H oxidase catalytic subunit].[11] In the present study, we demonstrated the presence of CyPA in atheromatous plaque in VSMC and EC, and extracellularly near the elastic lamina. Consistent with our results in VSMC, Dreher et al reported that CyPA was induced by oxidative stress in EC.[27] Taken together, these results suggest that CyPA is released and present during atherogenesis. There are few data regarding the levels of CyPA in human blood. Billich et al reported concentrations of CyPA in synovial fluids of patients with rheumatoid arthritis in the range of 11 to 705 nM, based on the enzyme activity.[13] We have developed an ELISA that shows a linear relationship between optical density and CyPA concentration over the range of 0.1 to 1000 nM. Using this ELISA, we found CyPA plasma levels of 5 to 100 nM in patients with unstable angina (Jin and Berk, unpublished data). Thus, the concentrations of CyPA used in this study are pathophysiologically relevant.

Previously, we demonstrated in VSMC that CyPA stimulated ERK1/2 activation, increased DNA synthesis, and inhibited apoptosis.[11] In the present study, we found that CyPA activated human EC in a manner similar to the proinflammatory cytokine TNFα.[26] The ability of CyPA to activate EC and to trigger NF-κB activation suggests a novel mechanism by which oxidative stress stimulates arterial inflammation and suggests CyPA is an inflammatory cytokine. Increasing evidence suggests that atherosclerosis is an inflammatory disease of the vessel wall characterized by monocyte infiltration in response to proatherogenic factors.[4] Adhesion molecules expressed in endothelial cells play an important role in monocyte recruitment to atherosclerotic lesions. In this study, we showed that CyPA induced E-selectin and VCAM-1 expression in endothelial cells. In addition, CyPA has chemotactic activity to neutrophils[18,19] and monocytes.[18] Although we do not have direct evidence for a role of CyPA in monocyte recruitment in atherosclerotic lesions in vivo, our data suggest that CyPA expressed and secreted in atherosclerotic lesion may contribute to recurrent inflammatory events leading to atherosclerosis progression.

In this study, we also found that the combination of CyPA and CHX triggered EC apoptosis. In contrast, in VSMC, CyPA augmented DNA synthesis and inhibited cell apoptosis.[11] These differing results reflect multiple functions of CyPA in different cell types. Our findings that CyPA did not induce EC apoptosis in the absence of CHX and CyPA stimulated NF-κB activation in EC suggest that CyPA may stimulate antiapoptotic gene expression though NF-κB activation similar to results with TNFα.[26] Further study is needed to understand the antiapoptotic and proapoptotic signaling of CyPA in EC.

In conclusion, this study demonstrates that CyPA activates EC in a manner that would promote atherogenesis. We propose that ROS generated locally (eg, by monocytes and VSMC) causes VSMC and possibly EC to release CyPA. Secreted CyPA now binds to EC and stimulates inflammation and apoptosis. These changes result in recruitment of additional inflammatory cells and atherosclerosis progression via a positive feedback mechanism.

Acknowledgments

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