Cyclophilin a regulates autophagy of macrophage through PI3K/Akt/mTOR signaling

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ABSTRACT

Cyclophilin A (CyPA) is a ubiquitously expressed protein and secreted in response to inflammatory stimuli, such as hypoxia, infection, and oxidative stress. Macrophage autophagy performs an essential role in atheroprotection during early atherosclerosis. However, the role of CyPA in macrophage autophagy remains undefined. By Western blot, it demonstrated that CyPA increased level of autophagic marker protein light chain (LC) 3II in dose- and time-dependent manners, with the peak appearing at 100 ng/mL CyPA after 6 h exposure. Immunofluorescence and electron microscopy also confirmed similar results by detecting autophagosomes in RAW264.7 cells. Finally, it demonstrated that CyPA can induce autophagy by inhibiting phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin pathway. Inhibition of PI3K/Akt by LY294002 augmented CyPA-induced autophagy. The current study shows that CyPA could induce RAW264.7 cell autophagy, and it may regulate autophagy through inhibiting the PI3K/Akt/mTOR signaling.

INTRODUCTION

Atherosclerosis (AS)-related cardiovascular diseases are leading causes of death and morbidity among adults in developed countries [1]. AS refers to imbalanced lipid metabolism and a maladaptive response to inflammation; it is a vasculature disease characterized by chronic inflammation of large- and medium-sized arteries [2]. Three types of cells participate in initiation and development of AS: macrophages, smooth muscle cells, and vascular endothelial cells. Macrophages play a crucial role in all stages of AS development. During AS evolution, changes in macrophages are closely related with autophagy, which is involved in occurrence and development of the disease [3]. Correspondingly, recent investigations demonstrated that macrophage autophagy is involved in a novel pathway through which these cells contribute to vascular diseases [4–7].

Autophagy is an evolutionarily conserved and controlled cellular catabolic process characterized by sequestration of cytoplasmic organelles within double-membrane vesicles and degradation by lysosomal enzymes [8]. This process includes an intracellular degradative system, in which cytoplasmic cargo is delivered into lysosomes for ultimate degradation and recycling. When cells are challenged with metabolic stress, such as nutrient depletion and oxidative stress, autophagy is activated to protect cells from dying. Autophagy is considered a mechanism promoting cell survival. Recent research emphasized the important role of autophagy in AS formation. Autophagy in macrophage plays a protective role in advanced AS [7]. Macrophage autophagy becomes dysfunctional, which can result in inflammation and accelerate progression of AS [6]. At present, no study can elucidate the mechanism by which autophagy affects pathological processes, such as inflammation; autophagy

can also be a novel potential therapeutic strategy for prevention and treatment of AS [9]. With enhancement by drugs or other methods, macrophage autophagy potentially suppresses progression of atherosclerotic plaques and reduces disease stability [10]. An autophagy marker protein, microtubule-associated protein 1 light chain (LC) 3, is used in most assays for autophagy as indicator of autophagy activity. Several molecular and cell signaling pathways are implicated in regulating autophagy; these pathways include autophagy-related gene family, beclin-1, mitogen-activated kinase (MAPK), and phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K-AKT-mTOR) pathways [11–14]. Among these pathways, mTOR is negatively regulated in autophagy.

Cyclophilin A (CyPA), which belongs to immunophilin family, is a ubiquitously distributed and evolutionarily well-conserved protein. CyPA was initially purified from bovine thymocytes and originally identified as primary cytoplasmic binding protein of potent immunosuppressive drug cyclosporin A (CsA) [15,16]. CyPA possesses peptidylprolyl cis–trans-isomerase activity, and it is believed to be a key molecule in a growing number of biological functions, including molecular chaperoning, protein folding, protein trafficking, immune modulation, cell signaling immune response, and transcription regulation [17–20]. Silencing CyPA can inhibit proliferation, prevent cell cycle, and induce autophagy of BGC-823 gastric cancer cells *in vitro* [21].

Our previous study indicated that CyPA can mediate ox-low-density lipoprotein (LDL)-induced activation and apoptosis in RAW264.7 cells by regulating autophagy [22]. However, the interaction between CyPA and macrophage autophagy remains unclear. In the present study, we aimed to explore the relationship between CyPA and RAW264.7 cells and to determine pathways involved in CyPA-mediated RAW264.7 cell autophagy.

METHODS

Cell culture

Murine macrophage RAW264.7 cell line was obtained from the Cell Bank of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Wisent, Montreal, Canada). Cultures were maintained at 37° C in a humidified 5% CO₂ atmosphere. To maintain cells in a resting state, cells were passaged every day in a relatively low-density as other half suspension cells.

Western blot analysis

Total proteins were harvested by rinsing treated cells with ice-cold phosphate-buffered saline (PBS) and

lysing in lysis buffer for 30 min at 4° C. To remove debris, whole cell lysates were collected after centrifugation at 12,000 rpm for 10 min at 4° C. Protein concentration was measured using bicinchoninic acid protein assay reagent kit (Thermo Fisher, 23227). An equal amount of protein was separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk or 3% bovine serum albumin for 2 h at room temperature. Afterward, membranes were incubated overnight with primary antibodies against CyPA, LC3A/B phosphorylated-PI3K (p-PI3K), phosphorylated-Akt (p-Akt), phosphorylated-mTOR (p-mTOR), β -actin, phosphorylated-extracellular-signal-regulated kinase (p-ERK), phosphorylated-p38 (p-p38), and phosphorylated-c-Jun N-terminal kinase (p-JNK) (2715,12741,4228P, 4060P,5536P, 8457, 9101P,4631P,4668P,CST,USA, respectively) at 1:1000 and then were incubated at 4°C. The next day, membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (7074, CST, USA) for 1 h at room temperature. Bound antibodies were detected with an Image Quant LAS 4000 Imager, and densitometric analysis was performed using ImageJ image processing program.

Immunofluorescence

RAW264.7 cells were seeded at 1×10^5 per well on 12-well plates and allowed to continually proliferate for 24 h. According to the 200:1 ratio of serum-free medium dilution enrichment of concentrated GFP-RFP-LC3 adenovirus (Hanheng, Shanghai, China), 400 μ L of diluent adenovirus was added to each well. After 2 h co-culture, washing with PBS for twice. DMEM containing 6% FBS was used in the next phase of experimental group. For the control group, the medium contained 6% FBS and DMEM. Experimental group was treated with CyPA as described as above. Subsequently, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, fluorescence microscopy was used to observe the number of GFP⁺RFP⁺ puncta per cell in three independent experiments, including at least 50 cells scored in random fields.

Electron microscopy

Raw264.7 cells were treated as indicated, fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 2 h at 4° C, and postfixed with 1% osmium tetroxide at 4° C in the same buffer containing 0.8% potassium ferricyanide. Afterward, samples were dehydrated in a graded series of ethanol, infiltrated with Epon resin for 2 days, embedded in the same resin, and aggregated at 60° C for 48 h. Ultrathin sections were obtained using a Leica Ultracut UC6 ultramicrotome (Leica Microsystems, Vienna) and mounted on Formvar-coated copper grids.

Sections were stained with 2% uranyl acetate in water and lead citrate. Twenty-five sections/cell line were observed in an H-7650 electron microscope (Hitachi, Tokyo, Japan).

Inhibitor assay

To explore effects of PI3K as upstream target of PI3K/Akt/mTOR signaling pathway on CyPA-induced autophagy in RAW264.7 cell growth inhibition, 20 $\mu\text{mol/L}$ LY294002 (a PI3K specific inhibitor) was pretreated prior to addition of 100 ng/mL CyPA and incubated for 6 h. As described above, immunoblotting was used to detect protein expression against p-PI3K and LC3II/I antibodies.

Statistical analysis

Data are expressed as means $\pm$ S.E. Significance of differences was examined by Student's *t*-test and the variance analysis of repeated measurements multivariate. A *p* value < 0.05 or < 0.01 was considered statistically significant.

RESULTS

CyPA induces autophagy in RAW264.7 cells in dose- and time-dependent manner

Cytosolic form of LC3 (LC3I) is conjugated to phosphatidylethanolamine to form LC3I phosphatidylethanolamine conjugate (LC3II), which is considered a specific marker for autophagic activity during autophagy. To determine whether CyPA can induce autophagy in RAW264.7 cells, we treated RAW264.7 cells with varying CyPA concentrations, which ranged from 1 ng/mL to 1000 ng/mL. Afterward, we detected LC3II in whole cell lysates. Response was dose-dependent at 100 ng/mL concentration, thereby causing maximum change in LC3II level (Figure 1A). In subsequent set of experiments, we treated RAW264.7 cells with CyPA at 100 ng/mL concentration for 1, 3, 6, 12, and 24 h. LC3II level was detected in whole cell lysates using Western blot analysis. CyPA caused no change in LC3II level after 1 h exposure. However, in 3, 6, 12, and 24 h exposure, substantial increase was observed, with LC3II expression peaking at 6 h exposure showing (Figure 1B). Therefore, all other experiments were performed using CyPA at 100 ng/mL concentration with 6 h exposure time. We further treated macrophage cells with CsA, a CyPA inhibitor. Autophagy expression was evidently reduced (Figure 1C).

CyPA promotes autophagosome formation in RAW264.7 cells

GFP-RFP-LC3 adenovirus was used to trace LC3II in RAW264.7 cells. We confirmed that 100 ng/mL CyPA can induce autophagy in immunofluorescence

(Figure 2A). Yellow punctate dots (GFP⁺ RFP⁺) indicated autophagosomes, and red (GFP⁻ RFP⁺) ones were autolysosome. Given that autophagy is characterized with autophagosome formation, we further examined ultrastructure of CyPA-treated and control RAW264.7 cells using electron microscopy. CyPA-treated RAW264.7 cells showed numerous double-membrane vacuoles. However, no autophagosome was observed in the control group (Figure 2B). Altogether, these results demonstrated that CyPA induced autophagy in RAW264.7 cells.

CyPA activates autophagy through PI3K/Akt/mTOR pathway in RAW264.7 cells

MAPK, PI3K/Akt, and mTOR signaling pathways are involved in molecular biological mechanisms by which autophagy occurs [23]. Western blot assay was used to investigate whether effect of CyPA on RAW264.7 cells occurred through this pathway. We observed that cells treated with CyPA showed decreasing expressions of p-PI3K and p-Akt in RAW264.7 cells but not p-ERK1/2, p-p38, nor p-JNK1/2 (Figure 3A). To further confirm that CyPA-induced autophagy occurred through the PI3/Akt signaling pathway, we utilized LY294002 treatment. RAW264.7 cells with CyPA and LY294002 significantly decreased expression of LC3-II compared with that treated with CyPA alone (Figure 3B). This phenomenon was also verified by fluorescence microscopy and electron microscopy (Figure 4A and 4B). These consequences indicated that CyPA-induced autophagy resulted from inhibited phosphorylation of PI3K/Akt pathway.

DISCUSSION

CyPA, an important inflammatory mediator in development of AS, is a potent chemoattractant for leukocytes, monocytes, and lymphocytes; within the plaques, macrophages can secrete large amounts of CyPA when stimulated by ROS [24–26]. Studies have shown highly expressed CyPA in advanced atherosclerotic lesions. In ApoE^{-/-} and ApoE^{-/-}CyP^{-/-} mice, CyPA increased uptake of LDL and promoted formation of foam cells [21]. Verheye *et al.* used electron microscope and immunohistochemical staining and confirmed that macrophages and smooth muscle cells exhibit autophagy in atherosclerotic plaques [27]. Our experiment showed that CyPA induced autophagy in macrophages. These findings strongly suggest CyPA is most likely to be broadly involved in the regulation of pro-inflammatory activity of macrophages, and CyPA signaling pathway is one of important pro-inflammatory pathways in ROS.

Autophagy, an essential metabolic process, can clear damaged or senescent organelles and maintain basal energy balance [28]. This process may play a critical and decisive role in various human physiological and pathophysiological processes, including immunity,

cancer, neurodegenerative disorder, and cardiovascular diseases [29]. Some studies determined protective actions of autophagy in AS [30–32]. Therefore, macrophage autophagy poses benefits when induced appropriately. Under normal circumstances, augmented formation of autophagosome, which is characterized by increased LC3-II conversion, occurs during induced cellular autophagy. The results of our experiment showed that RAW264.7 cells exhibit low autophagy expression in normal conditions. When cells were treated with CyPA, LC3II was augmented evidently compared with controls. We further observed autophagosome in immunofluorescence and electron microscopy and obtained the same results, showing consistency with those of Western blot analysis.

Hence, we confirmed that CyPA can induce autophagy in RAW264.7 cells. These findings suggest that CyPA is most likely to be involved in the regulation of autophagy of macrophages, and maybe CyPA is the key factor that links autophagy and inflammatory activity of macrophages. Notably, when we continually increased CyPA concentration, autophagy level decreased. Thus, detailed mechanism involved should be further explored.

mTOR is a key regulator of autophagic activity in eukaryotic cells. Based on this fact, our study clearly demonstrated the role of mTOR pathway in CyPA-mediated autophagy. Specifically, we showed that the level of phosphorylated mTOR, a negative regulator of autophagy, was significantly reduced with CyPA

Figure 1: Cyclophilin A (CyPA) induces autophagy in RAW264.7 cells in time- and dose- dependent manner. (A) RAW264.7 cells were exposed to different CyPA doses for 24 h and subjected to Western blot analysis with anti-light chain (LC) 3II/I antibody. (B) RAW264.7 cells were exposed to 100 ng/mL CyPA at indicated times and subjected to Western blot analysis with anti-LC3II/I antibody. (C) RAW264.7 cells were treated with cyclosporin A (CsA) (200 ng/mL) for 24 h. LC3 II expression significantly decreased compared with that in control. Detection of β-actin was used as measure of equal loading. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test.

treatment. It also showed potential involvement of PI3K/Akt/mTOR signaling pathway in CyPA-treated RAW264.7 cells; this role was further confirmed by the PI3K specific inhibitor LY294002. Induction of autophagy in CyPA-treated RAW264.7 cells provided evidence on accumulation of biochemical hallmark protein of autophagy, that is, LC3-II, which plays

pivotal roles in autophagosome formation. Our study also suggests that PI3K inhibitor LY294002, which blocked LC3II conversion, confirmed ability of CyPA to induce RAW264.7 cell autophagy. The mechanism concerning how CyPA regulates macrophage autophagy through PI3K/Akt/mTOR signaling pathways should be investigated in the future.

Figure 2: (A) Punctate LC3II dots in CyPA-treated RAW264.7 cells. Cells were traced with GFP-RFP-LC3 adenovirus, treated with 100 ng/mL CyPA for 6 h, and fixed with 4% paraformaldehyde. Cells were examined by fluorescence microscopy. Images were obtained using a fluorescence microscope. Representative images show LC3 staining in different groups of RAW264.7 cells infected with GFP-RFP-LC3 adenovirus. Quantitative data showed GFP⁺RFP⁺ puncta per cell and GFP⁺RFP⁺ puncta per cell in three independent experiments, including at least 50 cells scored in random fields. (B) Electron micrographs show ultrastructure of CyPA-treated RAW264.7 cells. Controls received no CyPA treatment but were prepared following the same process. The other group (CyPA) showed RAW264.7 cells treated with 100 ng/mL CyPA for 6 h. Arrows in electron micrograph denote presence of autophagosomes.

Figure 3: CyPA induced autophagy through phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in RAW264.7 cells. (A) For representative immunoblot analysis of phosphorylated PI3K (p-PI3K), p-Akt, phosphorylated mammalian target of rapamycin (p-mTOR), phosphorylated extracellular-signal-regulated kinase (p-ERK1/2), phosphorylated c-Jun N-terminal kinase (p-JNK), and phosphorylated p38 (p-p38) protein expression, RAW264.7 cells were treated with indicated concentration of CyPA for 6 h. (B) Cells were pretreated with 10 μ M of PI3K inhibitor, that is, LY294002, prior to 100 ng/mL CyPA treatment for 6 h. LC3II conversion was determined by Western blot analysis. Protein expression was normalized to respective total protein content. β -actin was used as loading control. Quantitative data represent means $\pm$ SD of three replicated independent experiments. * p < 0.05 and ** p < 0.01 compared with control group.

The current study shows that CyPA substantially increased levels of LC3-II in RAW264.7 cells, it suggested that CyPA induced autophagy in macrophages. Enhanced autophagy ability caused by combining CyPA treatment with autophagy inhibitor of PI3K, it suggested CyPA-

induced autophagy by suppression of PI3K/Akt/mTOR signaling pathway. In summary, CyPA regulates autophagy of macrophage through PI3K/Akt/mTOR signaling. Thus, CyPA may be a potential target for atherosclerosis therapy, perhaps especially in response to ROS stimulation.

Figure 4: CyPA induced autophagy through the PI3K/Akt signaling pathway in RAW264.7 cells. (A) Cells were pretreated with 10 μ M of LY294002 prior to 100 ng/mL CyPA treatment for 6 h. Representative images show LC3 staining in different groups of RAW264.7 cells infected with GFP-RFP-LC3 adenovirus. Cells were examined by fluorescence microscopy. Quantitative data showed GFP⁺RFP⁺ puncta per cell and GFP⁺RFP⁺ puncta per cell in three independent experiments, including at least 50 cells scored in random fields. (B) Electron micrographs show ultrastructure of CyPA-treated RAW264.7 cells. Results were similar to those in Western blot analysis. Arrows in electron micrograph denote the presence of autophagosomes. Scale bar represents 2 μ m. Data are the means $\pm$ SE of the number of autophagosome per cell from three independent experiments including at least 30 cells.

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