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Original Article

Oxidized low-density lipoprotein inhibits the degradation of cyclophilin A via the lysosome in vascular smooth muscle cells

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Abstract: Background: Cyclophilin A (CyPA) plays an important role in the progression of atherosclerosis. Additionally, it has been reported that lysosomal function is markedly impaired in atherosclerosis induced by oxidized low-density lipoprotein (ox-LDL). As the CyPA degradation pathway remains to be elucidated, we aimed to uncover the role of lysosomes and ox-LDL in the degradation of CyPA. Methods: We exploited RNA interference (RNAi) in combination with either the lysosomal inhibitor chloroquine (CQ) or the proteasomal inhibitor MG-132 to examine CyPA turnover. We also investigated the role of ox-LDL in lysosomal function and the CyPA degradation pathway and determined whether CyPA interacts with the selective autophagy adaptor p62. Results: CQ markedly reversed the CyPA downregulation induced by RNAi and increased intracellular levels of LC3 and p62. MG-132 significantly suppressed polyubiquitinated protein degradation but did not inhibit RNAi-induced CyPA downregulation. Additionally, neither CQ nor MG-132 influenced the gene-silencing efficiency of CyPA siRNA. Moreover, ox-LDL induced cytosolic accumulation of p62 was inconsistent with increased expression of LC3-II. Meanwhile, ox-LDL inhibited RNAi-induced downregulation of CyPA. Immunofluorescence indicated colocalization of endogenous CyPA with ubiquitin and with p62 in response to CQ treatment, and co-immunoprecipitation analysis confirmed interaction between CyPA and p62. Conclusion: CyPA is degraded by a lysosome-dependent pathway that may involve p62-mediated selective autophagy. Furthermore, ox-LDL modulates the degradation of CyPA via its inhibitory role in lysosomes, contributing to increased expression of CyPA in atherosclerotic plaques.

Keywords: Oxidized low-density lipoprotein, cyclophilin A, RNA interference, cycloheximide, lysosome, atherosclerotic plaques

Introduction

Cyclophilin A (CyPA) has been implicated in the pathophysiology of various cardiovascular diseases [1]. We and others have demonstrated increased expression of CyPA in atherosclerotic plaques [2-4]. Furthermore, deficiency of CyPA decreases atherosclerotic lesion burden in a mouse model of atherosclerosis [5]. These data suggest that transcriptional or post-transcriptional downregulation of intracellular CyPA expression may provide a therapeutic strategy to reverse the progression of atherosclerosis; however, the underlying mechanisms involved in the degradation of CyPA have not been established.

Proteolytic systems are critical cellular housekeeping processes that are essential for removal of damaged or unwanted organelles and protein aggregates. Moreover, both the autophagy-lysosome system and the ubiquitin-proteasome system play crucial roles in the quality control of proteins in atherosclerosis [6, 7]. Oxidized low-density lipoprotein (ox-LDL) not only serves as an important mediator of foam cell formation, but also exhibits functions involved in other atherogenic mechanisms [8]. It has been demon-

strated that ox-LDL increases lysosomal pH and lysosomal membrane permeability and diminishes the proteolytic capacity of lysosomes [9]. It remains uncertain whether the degradation pathway of CyPA is disturbed by ox-LDL, resulting in the accumulation of CyPA in atherosclerotic plaques.

Thus, to characterize CyPA degradation and determine whether ox-LDL mediates degradation of CyPA, we conducted a series of experiments in primary cultured rat aortic smooth muscle cells (RASMCs). Our results show that ox-LDL suppresses lysosome-dependent degradation of CyPA and that p62-mediated selective autophagy may be involved in CyPA degradation.

Materials and methods

Antibodies and reagents

The following primary antibodies were used: CyPA (Abcam, ab41684), SQSTM1/p62 (Abcam, ab56416), SQSTM1/p62 (Sigma, P0067), ubiquitin (Abcam, ab7254), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, 2118), and LC3 (Novus Biologicals, NB100-2220). Horseradish peroxidase (HRP)-conjugated secondary antibodies (sc-2004 and sc-2005) were purchased from Santa Cruz Biotechnology. Alexa Fluor 488 anti-rabbit and Alexa Fluor 647 anti-mouse secondary antibodies were purchased from Thermo Fisher Scientific. The proteasomal inhibitor MG-132 (S2619) was obtained from Selleckchem. The lysosomal inhibitor chloroquine (CQ; CC6628) was purchased from Sigma-Aldrich. Cycloheximide (CHX; C011) was obtained from MDBio, Inc., and ox-LDL (YB-002) was obtained from Yiyuanbiotech.

Cell culture

RASMCs (R6110, ScienCell; RA-6080, Cell Biologics) were cultured in Dulbecco's Modified Eagle Medium (DMEM) high-glucose (4.5 g/L; Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). Cells were cultured at 37°C in a humidified incubator (5% CO_2/95% air) as previously described [10]. RASMCs at passages 4-8 at 70% to 80% confluence were used for experiments.

In vitro treatments

To explore the effect of CHX on the translation of CyPA, RASMCs were incubated with 5 μg/mL CHX for the indicated times or treated with different concentrations of CHX (1.25-20 μg/mL) for 48 h. To evaluate the functions of the proteasome and the lysosome in response to CHX, cells were exposed to 5 μg/mL CHX combined with either MG-132 (0.1-10 μmol/L) or CQ (1.25-10 μmol/L) for 48 h.

To explore the effect of siRNA downregulation of CyPA, cells were transfected with 100 nmol/L CyPA siRNA or control siRNA for 6 h and then incubated in DMEM for the indicated times (0-72 h). To identify the role of either the lysosome or proteasome in the degradation of CyPA by RNA interference (RNAi), cells were transfected with CyPA siRNA or control siRNA for 6 h and then incubated with DMEM containing MG-132 (0.125-1 μmol/L) or CQ (1.25-10 μmol/L) for 48 h.

Furthermore, to determine the function of lysosomes in response to ox-LDL, RASMCs were incubated with different concentrations of ox-LDL (6.25-100 μg/mL) for 24 h. To explore the effect of ox-LDL on siRNA-induced degradation of CyPA, cells were transfected with 100 nmol/L CyPA siRNA or control siRNA for 6 h and then incubated with DMEM containing ox-LDL (12.5-100 μg/mL) for 48 h.

After treatment, RNA and protein lysates were extracted from the cells and analyzed by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) and western blotting, respectively.

Western blot analysis

Western blot analysis was performed as previously described [10]. Cells were lysed with radioimmunoprecipitation assay buffer (CST) supplemented with 1:100 Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). The total protein concentrations of the supernatants were determined using a colorimetric bicinchoninic acid protein assay kit (Pierce). Protein lysates were separated on sodium dodecyl sulfate-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked for 1 h at room temperature with 5%

w/v bovine serum albumin in Tris-buffered saline supplemented with 0.1% Tween-20. The membranes were probed with primary antibodies against CyPA (1:1,000), SQSTM1/p62 (1:1,000), ubiquitin (1:1,000), GAPDH (1:1,000), and LC3 (1:500) overnight at 4°C. Proteins of interest were detected with HRP-conjugated secondary antibodies (goat anti-rabbit IgG-HRP 1:5,000 and goat anti-mouse IgG-HRP 1:2,000). Proteins were visualized using an enhanced chemiluminescence detection system (Bio-Rad). The band intensity was analyzed using ImageJ software (NIH).

RT-qPCR

Total RNA was isolated from cells using the TRIzol reagent (Takara, 9109). Reverse transcription was performed on total RNA (0.5 μg) using PrimeScript RT Master Mix (Takara, RR036A). Then, qPCR was performed using SYBR Premix Ex Taq (Takara, RR420A) according to the manufacturer's instructions. The mRNA levels were normalized to the β-actin gene. The following primers were used in this study: 1) CyPA (GenBank accession number: NM_017101.1): forward 5'-TCAACCCCACCGTGTTCTTC-3', reverse 5'-AGCCAAATCCTTTCTCCCCA-3' and 2) β-actin (GenBank accession number: NM_031144.3): forward 5'-TCCTCCCTGGAGAAGAGCTA-3'; reverse 5'-TCCATACCCAGGAAGGAAGG-3'.

RNAi

Specific siRNA against rat CyPA mRNA (NM_017101.1; siRNA #1: ACGGAGAGAAAUUUGAGGAUGAGAA, UUCUCAUCCUCAAAUUUCUCUCCGU; siRNA #2: GGCUGGAUGGCAAGCAUGUGGUCUU, AAGACCACAUGCUUGCCAUCCAGCC; and siRNA #3: GGGAAGGUGAAAGAAGGCAUGAGCA, UGCUCAUGCCUUCUUUCACCUUCCC) were designed and synthesized by Invitrogen. Negative control Stealth RNAi siRNA (Invitrogen) acted as scrambled control siRNA. Based on the manufacturer's recommendation, RASMCs were transfected with 100 nmol/L of siRNA for 6 h using Lipofectamine RNAiMAX (Invitrogen) and were then co-incubated with or without different inhibitors for the indicated times. The cells were then lysed for experiments.

Immunofluorescence

After treatment with 10 μmol/L CQ, RASMCs were fixed and permeabilized with BD Fixation/Permeabilization solution for 20 min (BD Biosciences). Cells were washed thrice with 1× phosphate-buffered saline (PBS) and blocked with 5% bovine serum albumin in 1× Tris-buffered saline supplemented with 0.1% Tween-20 at room temperature for 30 min. Cells were probed with a mixture of anti-CyPA primary antibody (1:200) and anti-p62 primary antibody (1:100) or anti-ubiquitin primary antibody (1:100) overnight at 4°C. After washing with 1× PBS, cells were probed with Alexa Fluor 488 anti-rabbit or Alexa Fluor 647 anti-mouse secondary antibodies for 1 h at room temperature. Cells were washed thrice with 1× PBS, and then nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). All images were captured by confocal fluorescence microscopy (LSM710, Carl Zeiss).

Co-immunoprecipitation

RASMCs were lysed with CST buffer supplemented with 1:100 Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). For co-immunoprecipitation, protein extracts were incubated with rabbit polyclonal antibodies raised against p62 (Sigma) for 4 h at 4°C. Prewashed SureBeads protein A magnetic beads (Bio-Rad) were added to these samples and incubated for 1 h. Beads were washed, and immunoprecipitated proteins were eluted by incubated with 40 μl 1× Laemmli buffer (Bio-Rad) for 10 min at 70°C. Eluted immunoprecipitated proteins were analyzed by western blotting with CyPA antibody.

Statistical analysis

The data are expressed as mean ± standard error of mean (SEM). Comparisons were performed using one-way ANOVA followed by the Least Significant Difference post-hoc test. Statistical analyses were performed using GraphPad Prism software. Statistical significance was indicated by $P < 0.05$.

Results

CHX-chase immunoblotting is not suitable for identifying CyPA turnover

To characterize the degradation pathways of individual proteins, a lysosomal inhibitor and proteasomal inhibitor were combined with CHX to eliminate the added variable of *de novo* protein synthesis [11-14]. In CHX-chase immunoblotting experiments to examine the degradation

pathway of CyPA, CyPA protein levels were stably expressed in RASMCs during a 48-h CHX treatment when *de novo* protein expression was halted (**Figure 1A**). In contrast, the levels of polyubiquitinated proteins were clearly decreased within 1 h of CHX treatment (**Figure 1A**). In addition, we confirmed that CHX did not affect the degradative activity of the lysosome and the proteasome (**Figure 1B**, **1C**). A previous study demonstrated that CyPA protein levels were markedly downregulated after a 24-h RNAi treatment [15], indicating that spontaneous CyPA degradation occurred if *de novo* synthesis of CyPA protein was blocked by RNAi. Likewise, we confirmed that CyPA protein levels were significantly downregulated after 24-h CyPA RNAi treatment (**Figure 1D**). Our results indicate that CHX does not effectively inhibit protein translation of CyPA and therefore, CHX-chase assays are not suitable for investigations of CyPA turnover.

Degradation of CyPA occurs via the lysosome but not the proteasome

Transcriptional silencing of targeted mRNAs by siRNA is a specific method of suppressing the *de novo* synthesis of relevant proteins, and we confirmed that CyPA protein levels were specifically downregulated by targeted RNAi. Hence, we further exploited RNAi, in combination with either the lysosomal inhibitor CQ or the proteasomal inhibitor MG-132, to investigate the turnover of CyPA. CQ markedly reversed the CyPA downregulation induced by RNAi and led to increased intracellular levels of LC3 and p62 (**Figure 2A**). MG-132 significantly suppressed polyubiquitinated protein degradation but did not inhibit the CyPA protein downregulation induced by RNAi (**Figure 2B**), suggesting that the degradation of CyPA is specific to the lysosome. Furthermore, we examined the possibility that CQ treatment reversed siRNA-induced CyPA downregulation via weakening of the gene-silencing efficiency of the CyPA siRNA. We confirmed that neither CQ nor MG-132 reversed the ability of the CyPA siRNA to silence the expression of CyPA via mRNA analysis (**Figure 2C**). These data indicate that CyPA is degraded via a lysosome-dependent pathway in RASMCs.

Ox-LDL suppresses the degradation of CyPA via its inhibitory role in lysosomes

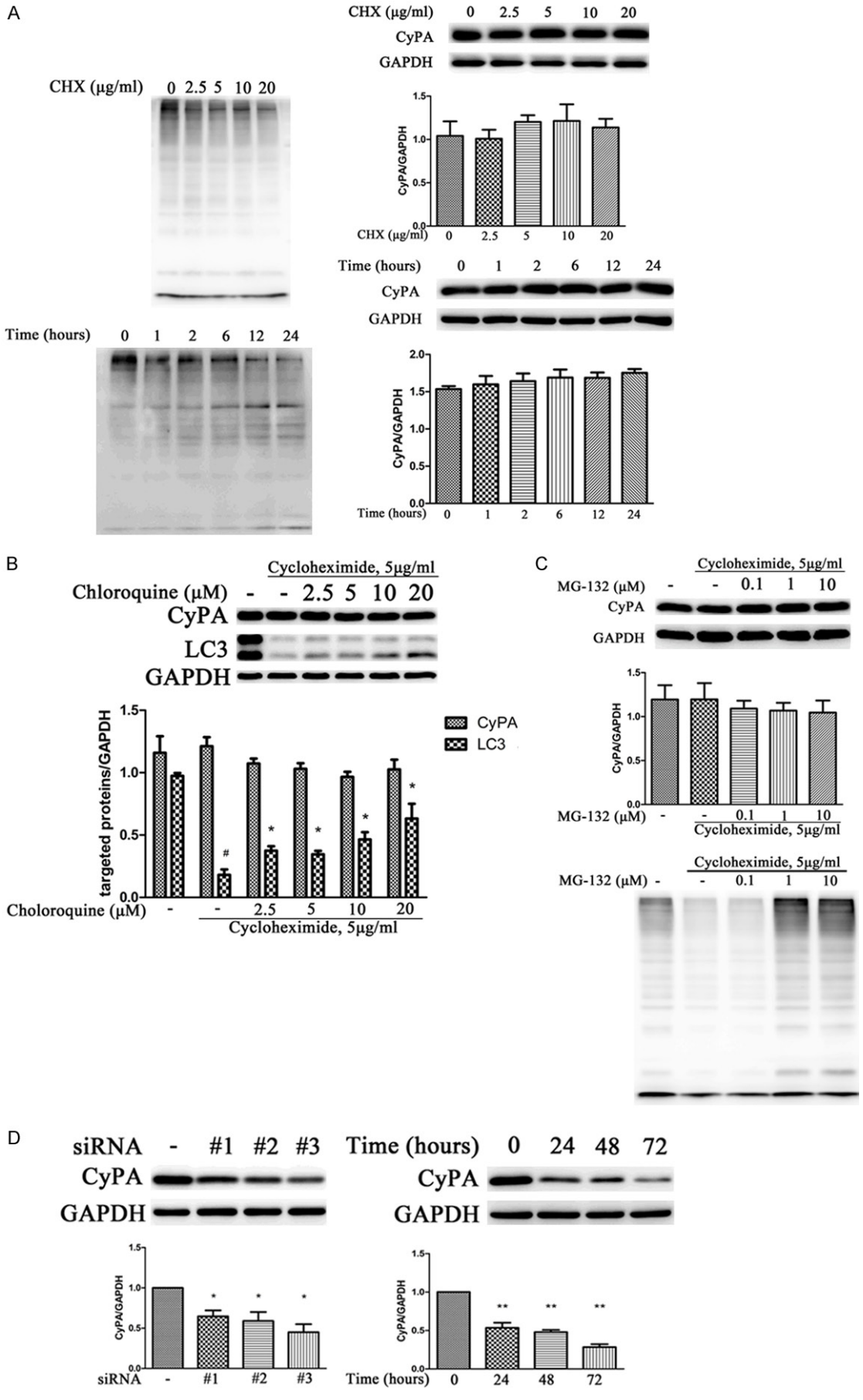
A previous study demonstrated that ox-LDL impairs lysosomal function in macrophages [9]. Therefore, we determined whether ox-LDL plays an inhibitory role in lysosomes in RASMCs. Indeed, ox-LDL induced cytosolic accumulation of p62 that was not consistent with increased expression of LC3-II (**Figure 3A**). Similar to previous results [9], the observation that p62 accumulates in the cytoplasm in the absence of consistent changes in LC3-II or autophagic flux suggests that ox-LDL does not inhibit autophagy per se, but rather inhibits the final component of the system - the lysosome. Additionally, we further identified the role of ox-LDL in the degradation of CyPA. As expected, ox-LDL significantly blunted the downregulation of CyPA induced by RNAi (**Figure 3B**). Our results indicate that ox-LDL suppresses the degradation of CyPA via its inhibitory role in the lysosome.

p62-mediated selective autophagy may be involved in the degradation of CyPA

The autophagic adaptor p62 recognizes ubiquitinated proteins for lysosome-dependent degradation [16], and a proteomic search of protein ubiquitination in murine tissues revealed that CyPA is likely to be ubiquitinated [17], although such modification of CyPA has not been directly verified. Therefore, we determined whether CyPA interacts with either p62 or ubiquitin. Using an immunofluorescence assay, we found that endogenous CyPA colocalized with either ubiquitin or p62 in response to CQ treatment (**Figure 4A**). In co-immunoprecipitation analysis, we confirmed that CyPA coprecipitated with p62 (**Figure 4B**). Our data demonstrate that p62-mediated selective autophagy may be involved in the degradation of CyPA.

Discussion

In the present study, we determined that CHX, in combination with either a lysosomal or proteasomal inhibitor, was not suitable for identifying the degradation pathway of CyPA. Using a combination of specific RNAi with either a lysosomal or proteasomal inhibitor, we further determined that the CyPA degradation pathway involves the lysosome. In addition, we demonstrated that ox-LDL impairs the degradative function of the lysosome, effectively suppressing the degradation of CyPA in RASMCs. Furthermore, we showed that p62-mediated selective autophagy may be involved in the degradation of CyPA.


A
CHX (μg/ml) 0 2.5 5 10 20
CyPA
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CyPA/GAPDH
Time (hours) 0 1 2 6 12 24
B
Cycloheximide, 5μg/ml
Chloroquine (μM) - - 2.5 5 10 20
LC3
targeted proteins/GAPDH
C
MG-132 (μM) - - 0.1 1 10
D
siRNA - #1 #2 #3
Time (hours) 0 24 48 72

Figure 1. CHX-chase immunoblotting is not suitable for examining CyPA turnover. A. Western blots of polyubiquitinated proteins and CyPA levels in RASMCs treated with different concentrations of CHX (1.25 to 20 μg/mL) for 48 h (top) or 5 μg/mL CHX for the indicated times (bottom). B. Western blots of CyPA and LC3 levels in RASMCs co-incubated with 5 μg/mL CHX and CQ (1.25 to 10 μmol/L) for 48 h. C. Western blots of CyPA levels and polyubiquitinated proteins in RASMCs co-incubated with 5 μg/mL CHX and MG-132 (0.1 to 10 μmol/L) for 48 h. D. Western blots of CyPA levels in RASMCs transfected with three siRNA duplexes for 6 h and subsequently cultured in complete medium without siRNA-lipid complex for 48 h (left). Western blots of CyPA levels in RASMCs transfected with 100 nmol/L siRNA #3 for 6 h and subsequently cultured in complete medium without siRNA-lipid complex for the indicated times (0 to 72 h; right). GAPDH levels were used for normalization. Bar graphs represent the mean ± SEM of three independent experiments. #$P < 0.05$ compared with scrambled control siRNA; *$P < 0.05$, **$P < 0.01$ compared with CyPA siRNA #3.

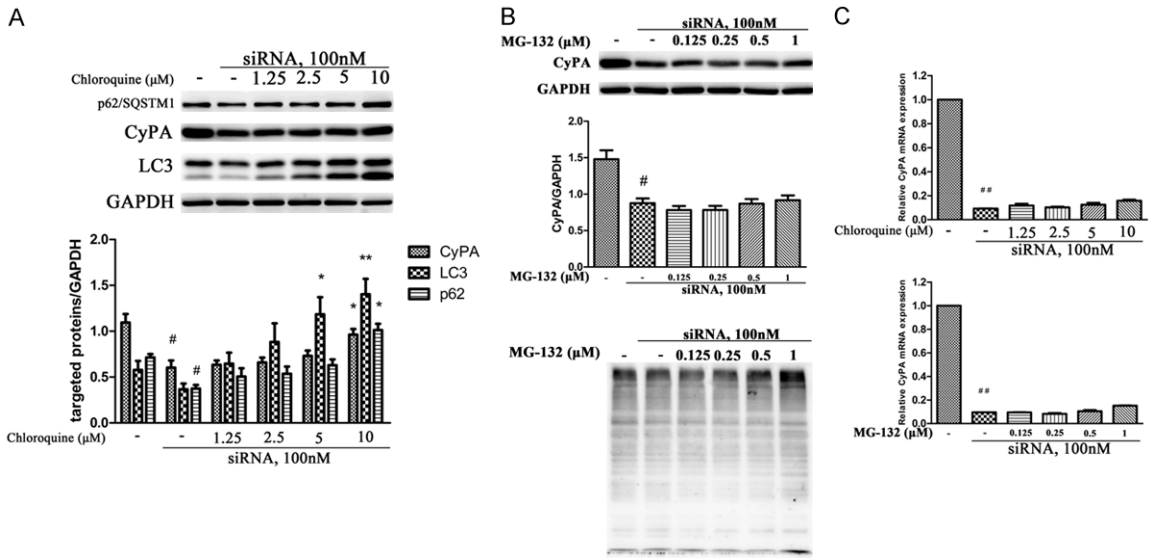


Figure 2. CyPA is degraded by the lysosome but not the proteasome, as determined by RNAi-chase immunoblotting. A. Western blots of p62, CyPA, and LC3 levels in RASMCs transfected with 100 nmol/L siRNA #3 for 6 h and subsequently cultured in DMEM with CQ (1.25 to 10 μmol/L) for 48 h. B. Western blots of CyPA levels and polyubiquitinated proteins in RASMCs transfected with 100 nmol/L siRNA #3 for 6 h and subsequently cultured in DMEM with MG-132 (0.125 to 1 μmol/L) for 48 h. C. RT-qPCR of CyPA mRNA levels in RASMCs transfected with 100 nmol/L siRNA #3 for 6 h and subsequently cultured in DMEM with either CQ (1.25 to 10 μmol/L) or MG-132 (0.125 to 1 μmol/L) for 48 h. GAPDH levels were used for normalization. Bar graphs represent the mean ± SEM of three independent experiments. #$P < 0.05$, ##$P < 0.01$ compared with scrambled control siRNA; *$P < 0.05$, **$P < 0.01$ compared with CyPA siRNA.

Based on the inhibitory effects of CHX on protein translation, the CHX-chase assay is widely applied to verify the stability of individual proteins [11, 18-20]. To eliminate the effects of *de novo* protein synthesis on the experimental results, a combination of CHX with either a lysosomal inhibitor or proteasomal inhibitor has been used to identify the degradation pathways of individual proteins [11-14]; however, the pellino E3 ubiquitin protein ligase family member 3 has been found to be stably expressed in the presence of CHX through an unknown mechanism [21]. Likewise, we observed that CyPA protein was stably expressed even after treatment with CHX, although CHX depletes cellular steady-state polyubiquitinated proteins [22, 23]. Our results agree with previous studies that showed that polyubiquitinated proteins are markedly decreased in the presence of CHX, indicating that CHX effectively inhibits protein translation and that steady-state proteins are subsequently spontaneously degraded. The CHX-chase assay is not suited for the examination of long-lived proteins due to the effects of prolonged protein synthesis inhibition on overall cell function [24]. As it has been reported that lysosomal protein clearance may be impaired by CHX treatment [25], we confirmed that CHX does not impair the degradative activity of either the lysosome or the proteasome in RASMCs. Together, these results suggest that CHX may not effectively

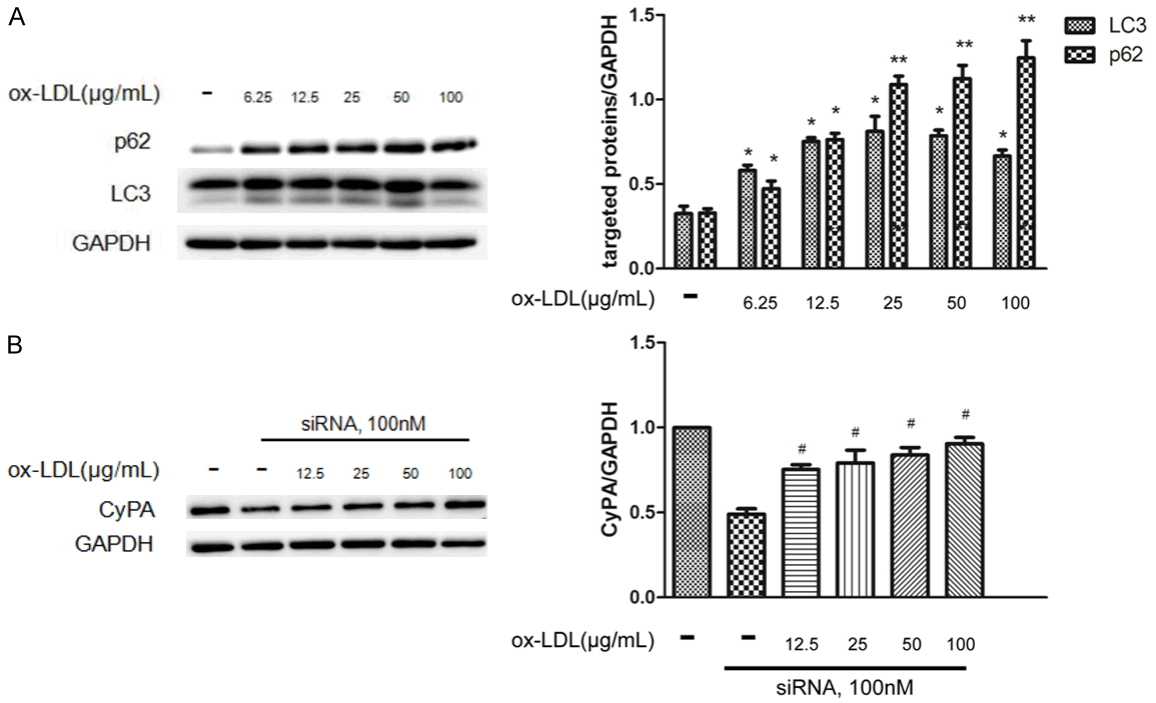
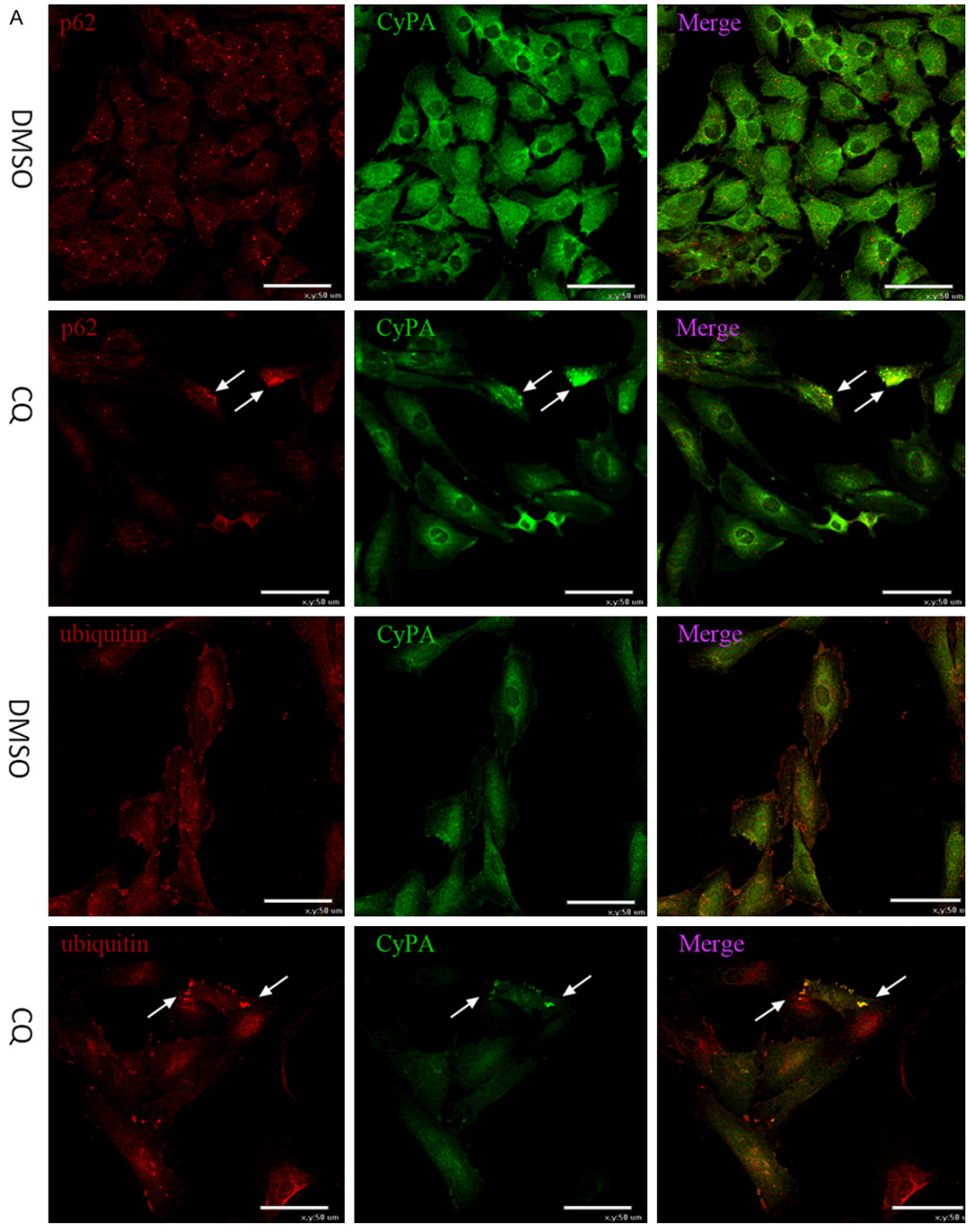


Figure 3. Ox-LDL suppresses the degradation of CyPA via its inhibitory role in lysosomes. A. Western blots of p62 and LC3 levels in RASMCs treated with ox-LDL (6.25 to 100 μg/mL) for 24 h. B. Western blots of CyPA levels in RASMCs transfected with 100 nmol/L siRNA #3 for 6 h and subsequently cultured in DMEM with ox-LDL (12.5 to 100 μg/mL) for 48 h. GAPDH levels were used for normalization. Bar graphs represent the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$ compared with untreated control; #$P < 0.05$ compared with CyPA siRNA.

inhibit the protein translation of CyPA and that the CHX-chase assay is therefore not suitable for the examination of CyPA turnover.

Using RNAi, we achieved suppression of CyPA protein synthesis. We confirmed that CyPA expression was significantly decreased in the presence of RNAi. Similarly, a previous study demonstrated that CyPA protein levels were significantly downregulated by RNAi within 24 h [15]. RNAi downregulates specific proteins by selectively cleaving the targeted mRNA without affecting the stability of irrelevant proteins. In our study, we confirmed that CyPA was gradually depleted when targeted mRNA was cleaved by specific RNAi. We further combined siRNA with either a lysosomal or proteasomal inhibitor to identify the degradation pathway of CyPA. Our results indicated that CyPA is degraded by the lysosome but not by the proteasome. CyPA is abundantly expressed in atherosclerotic lesions [2-4], but the source of CyPA in these atherosclerotic plaques remains unclear. It has been reported that lysosomal function is markedly impaired in atherosclerosis [9]. In fact, ox-LDL exhibits an inhibitory role in lysosomal function in macrophages [9]. Similarly, our studies revealed that ox-LDL inhibits the degradative function of lysosomes in RASMCs. In addition, we found that ox-LDL suppressed the depletion of CyPA protein induced by RNAi. These results demonstrate that degradation of CyPA via a lysosome-dependent pathway is suppressed by the lysosome inhibitory role of ox-LDL.

Successful lysosome-dependent degradation requires a concerted effort of autophagosome biosynthesis and cargo protein trafficking to the lysosome. Autophagic adaptor p62 recognizes ubiquitinated proteins for lysosome-dependent degradation by selective autophagy [16]. In our study, we found that CyPA interacts with p62 and may be ubiquitinated, suggesting that selective autophagy via p62 may be required for CyPA degradation. Altogether, our results indicate that CHX does not effectively inhibit CyPA protein translation and that CyPA is degraded via a lysosome-dependent pathway that may require p62-mediated selective


A
DMSO
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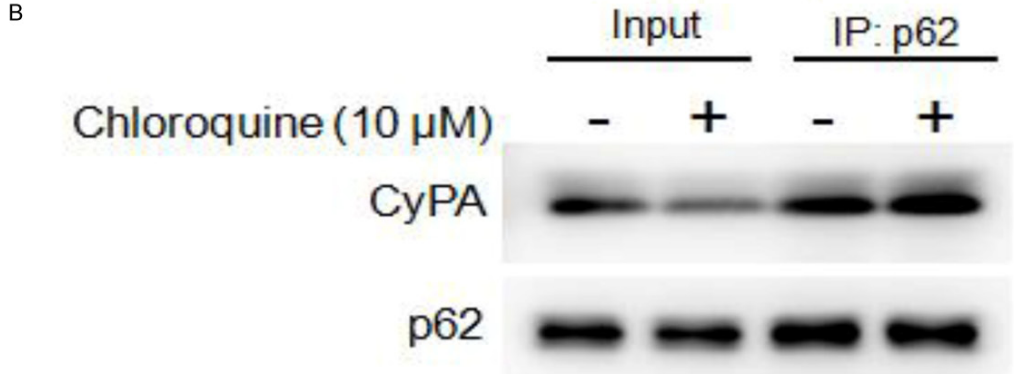
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Input
IP: p62
Chloroquine (10 μM)
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CyPA
p62

Figure 4. p62-mediated selective autophagy may be involved in the degradation of CyPA. A. Immunofluorescence assessing the colocalization of endogenous CyPA (green) with either p62 or ubiquitin (red) in response to 10 μmol/L CQ treatment. B. RASMCs were treated with 10 μmol/L CQ for 24 h, and proteins from cell lysates were immunoprecipitated with anti-p62 antibodies and immunoblotted with CyPA antibodies.

autophagy. Furthermore, these studies demonstrated that ox-LDL modulates the degradation of CyPA via its inhibitory role in lysosomes, perhaps contributing to the increased expression of CyPA observed in atherosclerotic plaques.

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Disclosure of conflict of interest

None.

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