의학박사 학위논문

C1q/TNF-related protein-9 이 자가포식 증가를 통해 혈관내피세포 노화에 미치는 영향

C1q/TNF-related protein-9 attenuates palmitic acid-induced endothelial cell senescence via increasing autophagy

울 산 대 학 교 대 학 원

의 학 과

이 지 우

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지 도 교 수　　박 중 열

이 논문을 의학박사 학위 논문으로 제출함

2021 년　2 월

울 산 대 학 교 대 학 원
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2021 년 2 월

Abstract

Background: Autophagy is an important process in the pathogenesis of atherosclerosis. C1q/tumor necrosis factor-related protein 9 (CTRP9) is the closest adiponectin paralog. CTRP9 has purported anti-aging and anti-atherogenic effects, but its roles in autophagy and endothelial senescence are unknown. The aim of this study was to evaluate whether CTRP9 prevents palmitic acid (PA)-induced endothelial senescence by promoting autophagy.

Methods: After no treatment or pre-treatment of human umbilical vein endothelial cells with CTRP9 prior to PA treatment, the level of senescence was measured by senescence associated acidic β-galactosidase staining and the level of hyperphosphorylated pRB protein. Autophagy was evaluated by LC3 conversion and the level of p62/SQSTM1, a protein degraded during autophagy. Autophagosome–lysosome fusion was detected by fluorescence microscopy.

Results: Pre-treatment with CTRP9 attenuated PA-induced endothelial senescence. CTRP9 increased the conversion of LC3-I to LC3-II, and decreased the level of p62 in time- and dose-dependent manners. Although both CTRP9 and PA treatment increased the LC3 conversion, treatment of PA increased p62 and decreased the fusion of autophagosomes and lysosomes, which represented decreased autophagic flux. However, pre-treatment with CTRP9 recovered the autophagic flux inhibited by PA. AMP-activated kinase (AMPK) activation was involved in LC3 conversion and decreased p62 induced by CTRP9.

Conclusion: CTRP9 inhibits PA-induced endothelial senescence by recovering autophagy and autophagic flux through AMPK activation.

Keywords: C1q/TNF-related protein-9, endothelial senescence, autophagy, aging, atherosclerosis

차 례

그 림 차 례

Introduction

Aging of endothelial cells is an independent risk factor of atherosclerosis-related diseases such as myocardial infarction, ischemic heart diseases, and stroke [1)]. Both aging and atherosclerosis are significantly associated with endothelial cellular senescence. Cellular senescence induces irreversible growth arrest of endothelial cells by the accumulation of nuclear and mitochondrial DNA damage and by increased levels of reactive oxygen species (ROS) [2, 3)]. In addition, endothelial cell senescence is also induced by proinflammation, high glucose concentration [4)], and genetic factors [5)]. Among these factors affecting endothelial senescence, lipotoxicity from free fatty acids (FFAs) is considered to lead endothelial senescence through increased oxidative stress [6)]. Notably, exposure to high concentrations of palmitic acid (PA), the most abundant saturated FFA in human plasma, promotes endothelial dysfunction and inhibits endothelium repair through the increase in inhibitor of nuclear factor-kappa-B kinase subunit beta (IKKβ) activity [7)], which is the main signaling pathway responsible for cellular senescence [8)].

C1q/tumor necrosis factor-related protein 9 (CTRP9) is a secreted multimeric protein of the C1q family. CTRP9 is the closest paralog of the insulin-sensitizing adipokine, adiponectin [9)]. It is predominantly expressed in adipose tissue. Adipose tissue plays central roles that releases various cytokines called adipocytokines[10)]. Previous studies have reported

that adipose tissue increase expression of bioactive molecules, including tumor necrosis factor-α (TNF- α) [11], plasminogen-activator inhibitor type-1 (PAI-1) [12], and resistin [13] that is known to mediate pathogenesis of atherosclerosis[10, 14, 15]. In addition to metabolic function of CTRP9 that overlap with those of adiponectin, CTRP9 has been studied because of its anti-atherogenic effects [16, 17]. In mouse studies, CTRP9 levels were associated with coronary atherosclerosis disease (CAD) [18], and treatment of CTRP9 inhibited neointimal formation, which is one of the processes that transform plaques into a vulnerable form that is prone to rupture [9]. In other studies, CTRP9 treatment decreased vascular inflammation [17] and improved endothelial dysfunction [19]. However, the possible effect of CTRP9 on cellular senescence has not been fully investigated.

Autophagy is a regenerative survival process that is involved in clearing long-lived or injured proteins and organelles. Autophagy also provides cells with alternate nutrients produced by the recycling of cellular proteins [20]. Decreased autophagic activity with aging and accumulation of intracellular damage has been reported to determine the level of cellular senescence [20, 21]. In this context, declining autophagic function causes the senescence of vascular endothelial cells, which has been strongly associated with the pathogenesis of cardiovascular disease (CVD) [22].

Presently, we examined whether CTRP9 inhibited endothelial senescence induced by lipotoxicity, such as PA. Considering that autophagy is an important preventive process of

cellular senescence, we hypothesized that autophagy and autophagic flux mediate this inhibitory effect of CTRP9 on endothelial senescence. In addition, because CTRP9 has been reported to attenuate endothelial inflammation mediated AMP-activated kinase (AMPK) signaling [17], we examined AMPK activation might mediate the effect of CTRP9 on autophagy induced inhibitory effects of cellular senescence.

Materials and Methods

1. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) obtained from Lonza Inc. (C2517A, Walkersville, MD) were cultured in endothelial basal medium (EBM-2, CC-3162, Lonza) supplemented with 2% fetal bovine serum (FBS) and various growth factors required for the growth of endothelial cells at 37℃ in a humidified incubator in an atmosphere of 5% CO2. Cells were used at six or less passages in all experiments. The cells were transferred to a medium containing 2% FBS and incubated for 24 hours in a medium containing 500 μM PA (P0500, Sigma-Aldrich, St. Louis, MO), the usual concentration applied in the endothelial cell research [23-26]. In all experiments, 2% bovine serum albumin was used as control.

CTRP9 (00081-04-100, Aviscera Bioscience Inc., Santa Clara, CA) and phosphate buffered saline was used as the vehicle. Cells were transformed to a medium containing 2% FBS and incubated in a medium containing various concentrations of CTRP9 for the indicated times before treatment with PA. To assess autophagic flux, HUVECs were treated with 10 nmol/L of bafilomycin A1, an inhibitor of vacuolar H+ adenosine triphosphatase (B1793, Sigma-Aldrich)

Compound C (AMP-activated protein kinase inhibitor, P5499, Sigma-Aldrich) was used as pre-treatment (10 μmol/L) to evaluate the involvement of AMPK signaling pathway

in autophagic induction by CTRP9.

2. Senescence associated acidic β-Galactosidase (SA-β-GAL) staining

Senescence was assessed by SA-β-GAL staining with cellular senescence staining kit (9860, Cell Signaling Technology, Danvers, MA) according to the manufacturer's protocol [27].

3. Western blot analysis

After lysis of cells, protein samples (20 μg/lane) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (RPN303D, GE Healthcare Life Sciences, Piscataway, NJ). Membranes were incubated in blocking buffer and then with one or more of the following primary antibodies: anti-AMPK (2532, Cell Signaling Technology), anti-hyperphosphorylated retinoblastoma gene product (ppRB, 9308, Cell Signaling Technology), anti-LC3B (2775, Cell Signaling Technology), anti-SQSTM1/p62 (sequestosome 1, 5114, Cell Signaling Technology), anti-autophagy-related genes 7 (ATG7, 2631, Cell Signaling Technology), and anti-ATG12 (2010, Cell Signaling Technology). All antibodies were used at a 1:1000 dilution. Mouse monoclonal antibodies used as loading controls at 1:10,000 dilution included anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH, 2118, Cell Signaling Technology) and anti-β-actin (A5441, Sigma-Aldrich). After incubating with primary antibodies, membranes were washed

and incubated with horseradish peroxidase-conjugated secondary antibodies (PI-1000, PI-2000, Vector Laboratories, Burlingame, CA). Immunoreactive bands were visualized by enhanced chemiluminescence (NEL103001EA, PerkinElmer, Waltham, MA).

4. Real-time PCR analysis

cDNAs were synthesized using the ReverTra Ace qPCR RT kit (FSQ-101, Toyobo, Osaka, Japan). Real-time analysis was conducted on an ABI 7500 Fast RT-PCR system with the Fast SYBR® Green Master Mix (4385612, Applied Biosystems, Foster City, CA). Each sample was assayed in duplicate in a 20 μL reaction volume containing 1 μL cDNA (corresponding to 100 ng of total RNA input), 10 μL of 2× SYBR Green Master Mix (4309155, Applied Biosystems), and 1 μL of forward and reverse primers (10 pmol/μL for each). Negative controls (no template or RNA) were included to ensure the absence of contamination. Amplification of 18S rRNA was used as the internal control. The ratio between the expression levels of the target gene and 18S rRNA was calculated using a relative quantification method (ΔΔ cycle value (Ct) method) as described previously (User Bulletin No. 2, Applied Biosystems). In brief, the amplification plot is a plot of fluorescence versus PCR cycle number. The threshold Ct is the fractional PCR cycle number at which the fluorescent signal reaches the detection threshold. Therefore, the input cDNA copy number and Ct are inversely related. Data were analyzed using the Sequence Detector System software version 2.1 (ABI) and the

Ct value was automatically converted to the fold-change RQ value. The fold-change (RQ) = $2^{-(\Delta\Delta CT)}$, where $-(\Delta\Delta CT) = -(\Delta CT_{trt} - \Delta CT_{control}) = -[(Ct_{Target} - Ct_{18s})_{trt} - (Ct_{Target} - Ct_{18s})_{control}]$. The following primers were used: ATG7, 5'-GTCCAAACCACCCTGTGG CA-3' (forward) and 5'-TGAATCTCCTGGGGCCACCA-3' (reverse); ATG12, 5'-TTGCTG CTGGAGGGGAAGG A-3'(forward) and 5'-CGCCAGCAGGTTCCTCTGTT-3' (reverse); and 18S 5'-CG CCGCTAGAGGTGAAATTC-3' (forward) and 5'-TTGGCAAATGCTTTCGCTC-3' (reverse).

5. Transfection of small interfering RNA (siRNA)

siRNA for human LC3 (also known as microtubule-associated protein 1 light chain 3 alpha, MAP1LC3A) and adiponectin receptor 1 (AdipoR1) were synthesized by Bioneer (Daejeon, Korea) using the targeting sequence 5'-CAUAAAGACACCCCUCAAA-3' for LC3 and 5'-CUCAUCAGAUUUUCCAUGU-3' for AdipoR1, respectively. For the experiments using siRNA, 50 nmol/L of targeted siRNA or 50 nmol/L of control siRNA (SN1013, Bioneer) were transfected into HUVECs using Lipofectamine 2000 (11668, Invitrogen, Life Technologies, Grand Island, NY) 24 hours prior to CTRP9 treatment.

6. Immunofluorescence

HUVECs were co-transfected with constructs encoding green fluorescent protein fused

to the N-terminus of LC3 (GFP-LC3) reporter and lysosome-associated membrane protein 1-tagged with red fluorescent protein (RFP-LAMP1) using Lipofectamine 2000. Coverslips were adhered to glass slides with fluorescence Mounting Medium (S3023, Dako, Carpinteria, CA). Colocalization of autophagy marker GFP-LC3 and RFP-LAMP1 was observed by laser scanning confocal microscopy using an LSM 780 microscope (Carl Zeiss, Oberkochen, Germany). Images were analyzed using Zen software (v2012, Carl Zeiss). Pearson's coefficient was used to measure the correlation between the signals from the two different marker proteins. Mander's coefficient was used to calculate the coefficient of overlap as previously described [28].

7. Statistical analyses

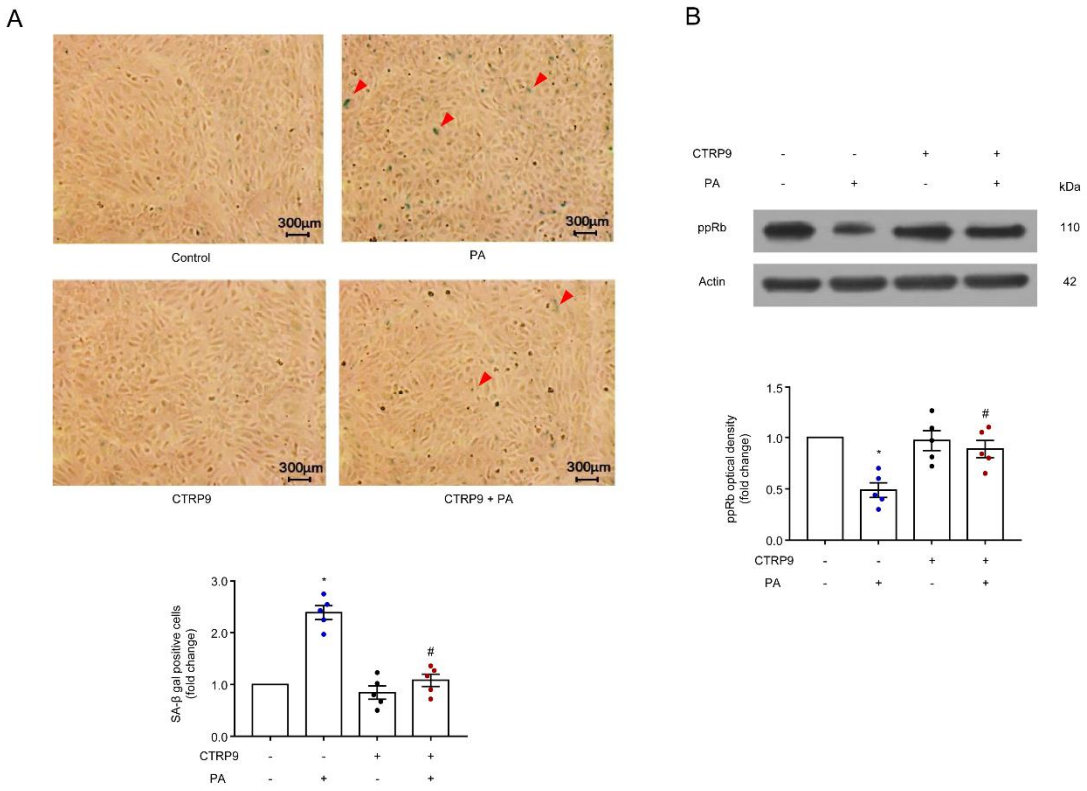
All data are presented as the mean ± SEM (standard error of the mean). Student's t-test was used to compare two groups. One-way analysis of variance (ANOVA) followed by a post-hoc analysis using the Tukey's multiple comparison test were used to compare multiple groups. P-values < 0.05 were regarded as statistically significant. All experiments were conducted at least five times. All statistical analyses were performed using SPSS 21.0 for Windows (IBM SPSS, Chicago, IL).

Results

1. CTRP9 attenuates PA-induced endothelial cell senescence

HUVECs were treated with 500 μM of PA for 24 hours to examine the effects of PA on endothelial senescence. The number of SA-β-Gal-positive cells was increased by treatment with PA (Figure 1A, right upper). Treatment only with CTRP9 did not change the frequency of SA-β-Gal-positive cells compared to controls (Figure 1A, left lower). However, when HUVECs were pre-treated with 3 μg/mL of CTRP9 for 1 hour before PA treatment, the number of SA-β-Gal-positive cells was significantly decreased compared to the cells treated with PA alone (Figure 1A, right lower). These results indicated that CTRP9 treatment can decrease PA-induced endothelial senescence in HUVECs. In addition, as senescence arrest in vascular endothelial cells are related to reduced levels of the hyperphosphorylated form of the retinoblastoma gene product (pRB) [28], the hyperphosphorylated pRB (ppRB) protein level was measured as an indicator of senescence. After PA treatment, the expression of ppRB decreased. However, pre-treatment with CTRP9 considerably increased the level of ppRB (Figure 1B).

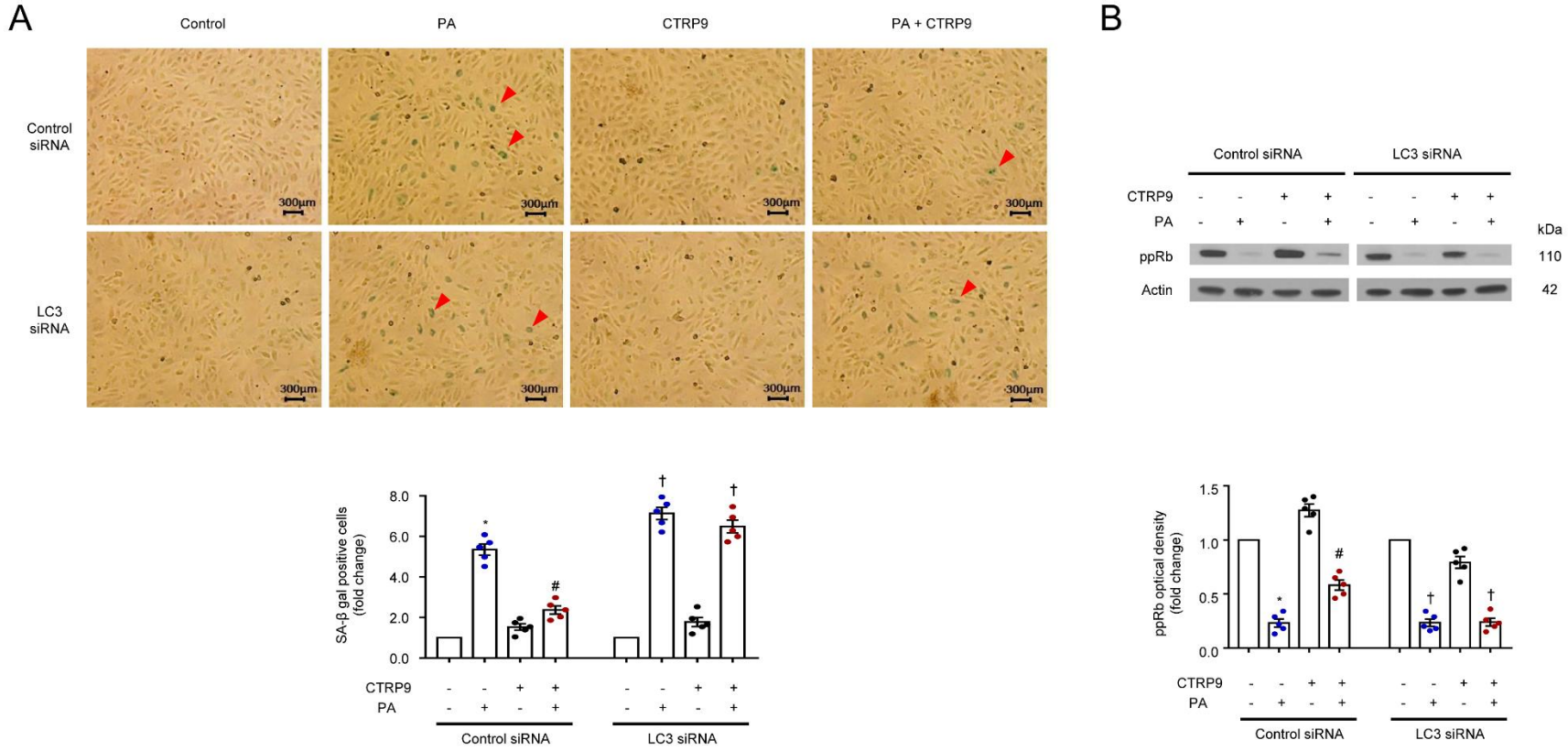
Figure 1. CTRP9 prevents PA-induced senescence in HUVECs. (A–B) SA-β-Gal staining (A) and protein level of ppRB (B) were used to evaluate the effect of CTRP9 on PA-induced endothelial senescence. The SA-β gal positive cells were stained green, indicated by arrowheads (representative cells). Data are presented as the mean ± SEM of five independent experiments. (*P <0.05 vs. untreated cells; #P <0.05 vs. cells treated with PA alone).



2. Inhibition of autophagy alleviates preventive effect of CTRP9 on PA-induced endothelial senescence

Next, to assess whether autophagy was involved in the inhibition of PA-induced endothelial senescence by CTRP9, HUVECs were transfected with LC3 siRNA or control siRNA. The decreased expression SA-β-Gal positive cells, demonstrating the preventive effect of CTRP9 against PA-induced endothelial senescence, was attenuated in LC3 siRNA-transfected cells (Figure 2A). Similarly, the increased expression of ppRb by CTRP9 was attenuated in LC3 siRNA-transfected cells (Figure 2B). Collectively, these results suggested that autophagy might be involved in the CTRP9's anti-senescent effect in vascular endothelial cells.

Figure 2. CTRP9 can abolish PA-induced endothelial cell senescence by increasing autophagy. (A–B) HUVECs were transfected with 50 nmol/L of LC3 or control siRNA. The SA-β-Gal positive cells (A) and the protein level of ppRB (B) were measured after treatment with 3 μg/mL CTRP9 in the presence or absence of 500 μM PA. The SA-β gal positive cells were stained green, indicated by arrowheads (representative cells). Data are presented as the mean ± SEM of five independent experiments. (*P <0.05 vs. untreated cells; #P <0.05 vs. cells treated with LA alone; †P <0.05 vs. cells treated with LC3 siRNA alone).


A
Control
PA
CTRP9
PA + CTRP9
Control siRNA
LC3 siRNA
300μm
SA-β gal positive cells (fold change)
8.0
6.0
4.0
2.0
0.0
CTRP9
PA
Control siRNA
LC3 siRNA
B
Control siRNA
LC3 siRNA
CTRP9
PA
kDa
ppRb
110
Actin
42
ppRb optical density (fold change)
1.5
1.0
0.5
0.0
CTRP9
PA
Control siRNA
LC3 siRNA

3. CTRP9 promotes autophagy and autophagic flux in HUVECs

To confirm the effects of CTRP9 on autophagy and autophagic flux, we measured protein expression of LC3-I/ LC3-II and p62/SQSTM1. The conversion of LC3-I to LC3-II, reflecting autophagosome formation [29], was estimated to assess whether CTRP9 increased the level of autophagy in time- and dose-dependent manners. The time- and dose- dependent protein level of p62, an indicator of autophagic flux [30], was also estimated to determine the effect of CTRP9 on the level of autophagic flux. When HUVECs were treated with 3 μg/mL of CTRP9 for 4–24 hours, conversion of LC3-I to LC3-II increased and p62 expressions decreased over time (Figure 3A). Likewise, CTRP9 increased LC3 conversion and decreased p62 expression in a dose-dependent manner (Figure 3B). These findings indicated that CTRP9 increased autophagy as well as autophagic reflux.

Additionally, we measured the mRNA and protein levels of ATGs involved in autophagosome formation. As previously reported[31], autophagic elongation needs two ubiquitin-like conjugation system. The ubiquitin-like protein ATG12 is conjugated to ATG5 by ATG7 and ATG10 enzymes, which participates in elongation autophagic membrane. A second conjugation system requires conversion of LC3-I to LC3-II, a key regulatory step in autophagosome formation, which occurs from the sequential action of ATG7 and ATG3 activities. To determine CTRP9 stimulates autophagosome formation, the mRNA expressions and protein levels of ATG levels (ATG7 and ATG12) were measured by real-time PCR analysis

and western blot analysis, respectively. Both mRNA and protein levels of ATG7 and ATG12 were increased by CTRP9 treatment in time- (Figure 4A) and dose-dependent manners (Figure 4B).

Figure 3. CTRP9 increases autophagy and autophagic flux. (A–B) HUVECs were incubated at 37°C with 3 μg/mL CTRP9 for different times (A), or with different concentrations of CTRP9 or control vehicle for 24 hours (B). The protein expressions of LC3-II/LC3-I and p62 were determined by western blotting using specific antibodies against LC3-II/LC3-I and p62. Data are presented as the mean ± SEM of five independent experiments. (*P <0 .05 vs. untreated cells; #P < 0 .05 vs. cells treated with CTRP9 for 4 hours; †P < 0.05 vs. cells treated with 0.3 μg/mL of CTRP9).

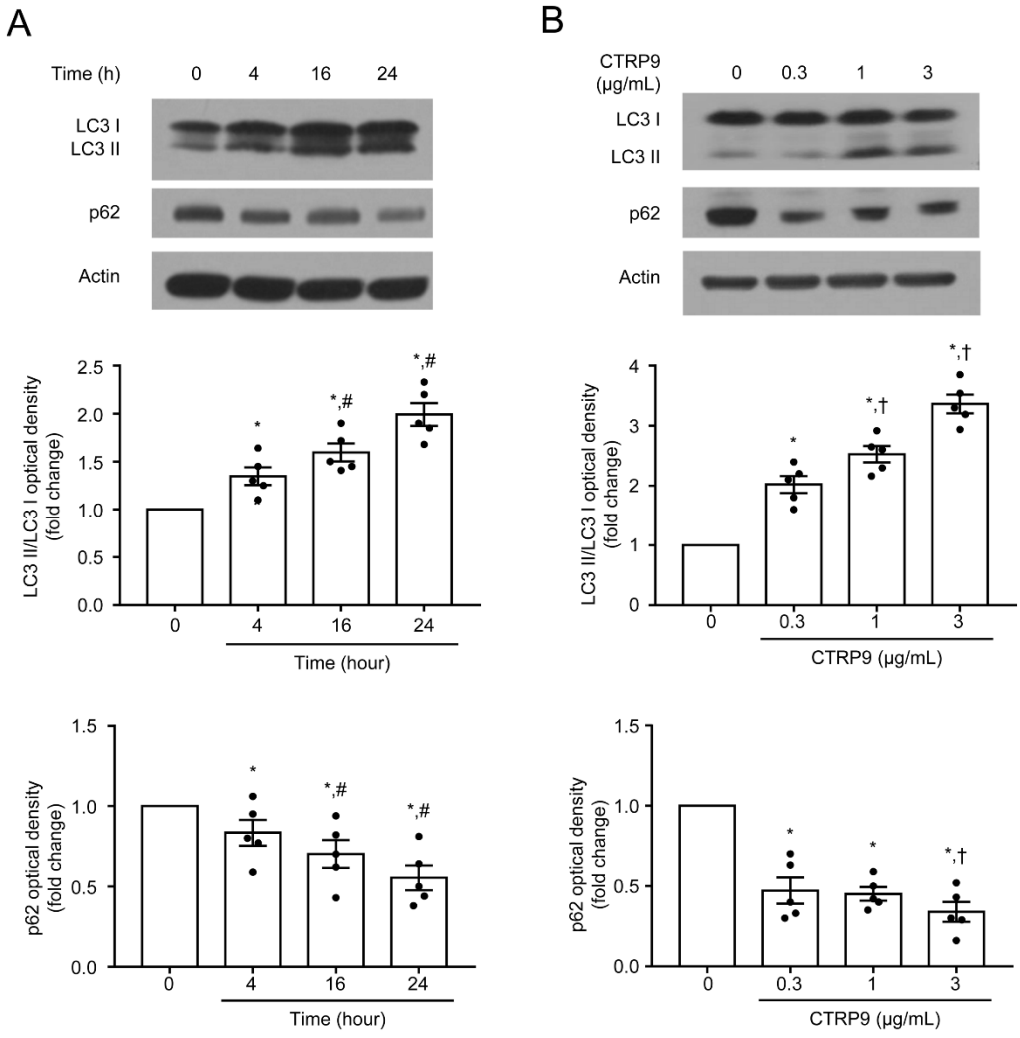
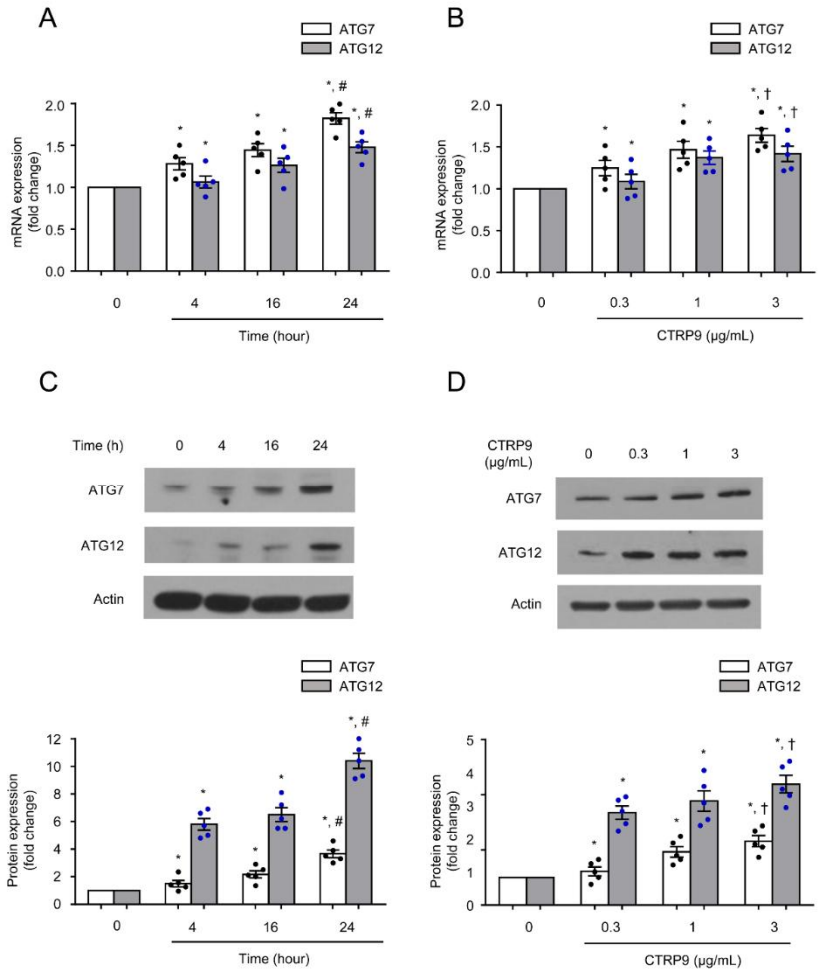


Figure 4. CTRP9 increases the expression of the autophagy-related gene, ATG. (A, B) After HUVECS were treated at 37°C with 3 μg/mL CTRP9 for different times (A), or with different concentrations of CTRP9 for 24 hours (B), the mRNA expression of ATG7 and ATG12 was measured by real-time PCR. (C, D) The protein levels were measured by western blot analysis. Data are presented as the mean ± SEM of five independent experiments. (*P <0 .05 vs. untreated cells; #P <0 .05 vs. cells treated with CTRP9 for 4 hours; †P < 0.05 vs. cells treated with 0.3 μg/mL of CTRP9).



4. CTRP9 recovers autophagy and autophagic flux inhibited by PA

Based on the findings that CTRP9 promoted autophagy and its flux in vascular endothelial cells, we next evaluated whether CTRP9 affected PA-induced changes in autophagic process in HUVECs. Both CTRP9 and PA treatment increased the LC3 conversion, but PA treatment increased p62 level, indicating the reduction of autophagic flux (Figure 5A). Distinct from PA treatment, CTRP9 prior to PA treatment decreased p62 level, suggesting that CTRP9 increased autophagy and also restored autophagic flux. Furthermore, the autophagic flux was examined by the treatment with bafilomycin A1, a pharmacological inhibitor of the fusion between autophagosomes and lysosomes [32]. After treatment with bafilomycin A1, CTRP9 treatment further increased LC3 conversion compared with cells treated with PA alone (Figure 5B).

To verify the fusion of the autophagosome and the lysosome, HUVECs were co-transfected with GFP-LC3 and RFP-LAMP1 and visualized by fluorescence microscopy. The fusion between autophagosomes and lysosomes was shown by the numbers of colocalized yellow foci, which were significantly increased in the CTRP9 treatment group compared with the PA group (Figure 6). The colocalization coefficients also demonstrated an increasing trend in the CTRP9 treatment group in the same manner (Figure 6).

Figure 5. PA-dependent inhibition of the autophagic machinery can be de-repressed by CTRP9. (A) The protein levels of LC3-II/LC3-I and p62 were measured by western blot analysis after treatment with 3 μg/mL CTRP9 in the presence or absence of 500 μM of PA ($^{*}P$ <0.05 vs. untreated cells; $^{\#}P$ <0.05 vs. cells treated with PA alone). (B) HUVECs were pre-treated with 10 nmol/L bafilomycin A1 and treated with 3 μg/mL CTRP9 in the presence or absence of 500 μM PA. The protein expressions of LC3-II/LC3-I were determined by western blotting analysis. Data are presented as the mean ± SEM of five independent experiments. ($^{*}P$ <0.05 vs. cells not treated with bafilomycin; $^{\dagger}P$ <0.05 vs. cells treated with bafilomycin A1 alone, $^{\S}P$ <0.05 vs. cells treated with bafilomycin A1 and PA).

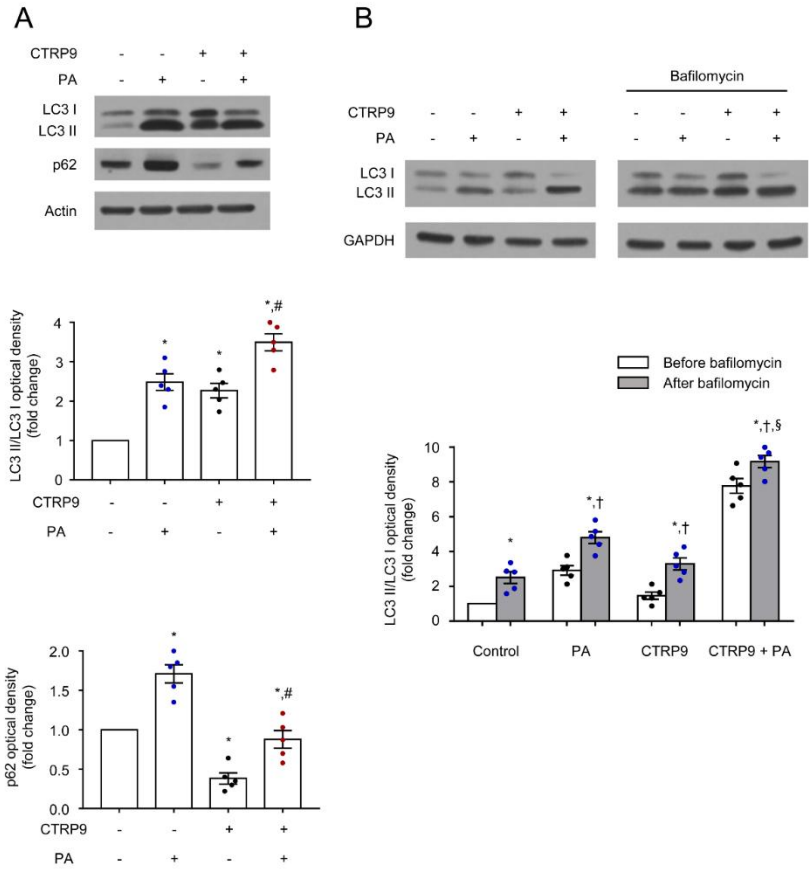
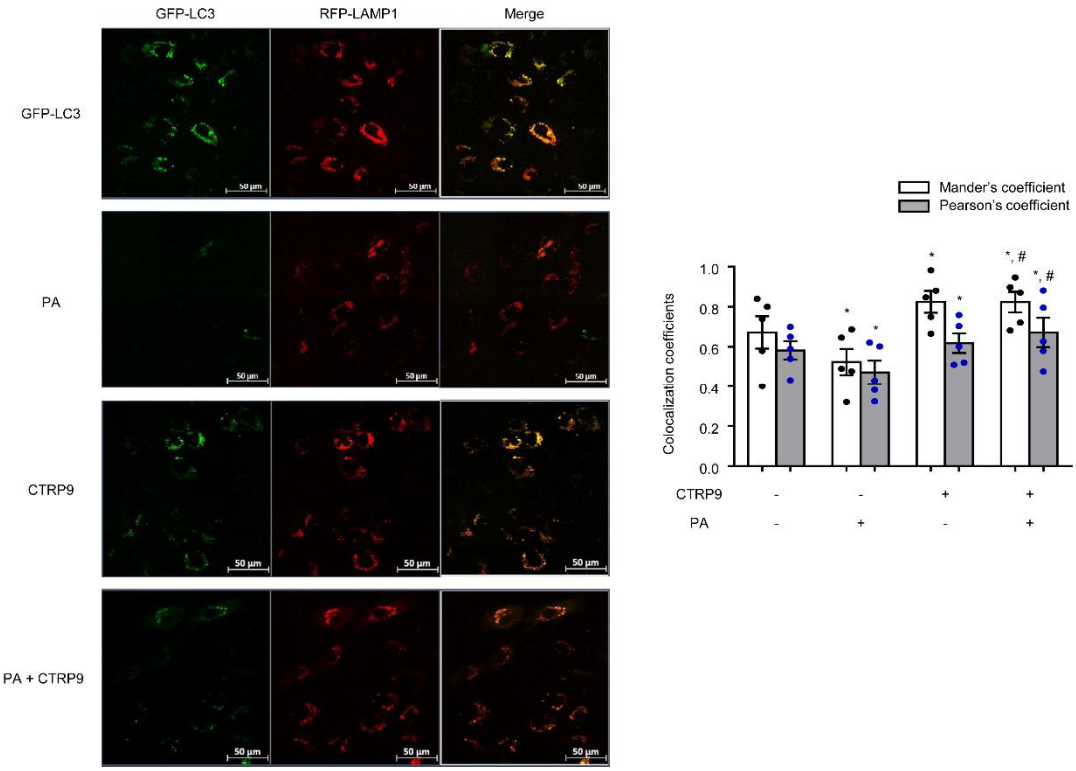


Figure 6. PA inhibits fusion of autophagosomes and lysosomes, whereas CTRP9 enhances formation of autophagosomes. After treatment with 3 μg/mL CTRP9 in the presence or absence of 500 μM PA, HUVECs were transfected with constructs encoding GFP-LC (green fluorescent protein fused to the N-terminus of LC3) and RFP-LAMP (lysosome-associated membrane protein 1-tagged with red fluorescent protein), and visualized by fluorescence microscopy. Mander's and Pearson's coefficients were used to assess colocalization. Data are presented as the mean ± SEM of five independent experiments. ($^{*}P$ <0.05 vs. untreated cells; $^{\#}P$ <0.05 vs. cells treated with PA alone).

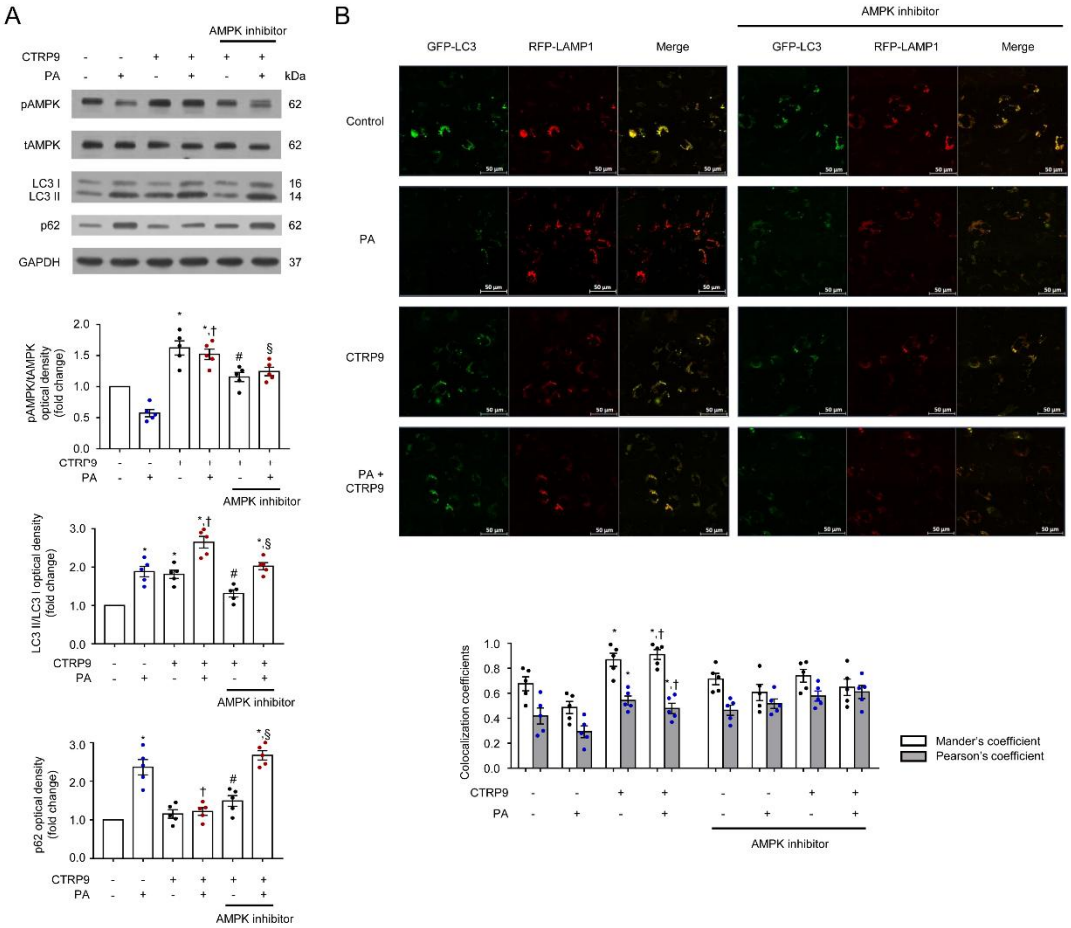


5. CTRP9 promotes autophagy and autophagic flux via AMPK pathways

The mechanisms of the autophagic process have been intensively studied. Several protein kinases are considered to be involved in this process [27]. Among these, the activation of AMPK has been reported to mediate the initiation of the autophagy process [33]. Furthermore, considering the important role of AMPK activation by CTRP9 in its cellular effect [17, 24, 33, 34], we measured the levels of phosphorylated AMPK, and treated HUVECs with compound C, a specific AMPK inhibitor. As previously reported [17, 24, 33, 35], CTRP9 increased the levels of phosphorylated AMPK (Figure 7A). Furthermore, this increased phosphorylation of AMPK by CTRP9 was maintained even under PA-stimulation (Figure 7A).

After inhibiting AMPK activity with a compound C, the CTRP9-dependent increase in LC3 conversion and decrease in p62 expression were attenuated (Figure 7A). In addition, HUVECs were pre-treated with the AMPK inhibitor and colocalized with GFP-LC3 and RFP-LAMP1 to demonstrate the role of AMPK activation on the fusion of the autophagosome and the lysosome. The inhibition of AMPK decreased the formation of autophagolysosomes induced by CTRP9 (Figure 7B). Increased colocalization coefficients induced by CTRP9 treatment were also abolished after the treatment of AMPK inhibitor (Figure 7B).

Figure 7. The AMPK signaling pathway is involved in CTRP9-induced autophagy in HUVECs. (A) The protein levels of AMPK and its phosphorylated forms were analyzed. The protein levels of LC3-II/LC3-I and p62 after pre-treatment with AMPK inhibitor (Compound C) followed by treatment with 3 μg/mL CTRP9 in the presence or absence of 500 μM PA. (B) The colocalization pattern of GFP-LC and RFP-LAMP. Mander's and Pearson's coefficients were calculated for colocalization analysis. Data are presented as the mean ± SEM of five independent experiments. ($^{*}P$ <0.05 vs. untreated cells; $^{\#}P$ <0.05 vs. cells treated with CTRP9 alone; $^{\dagger}P$ <0.05 vs. cells treated with PA alone; $^{\S}P$ <0.05 vs. cells treated with CTRP9 and PA).

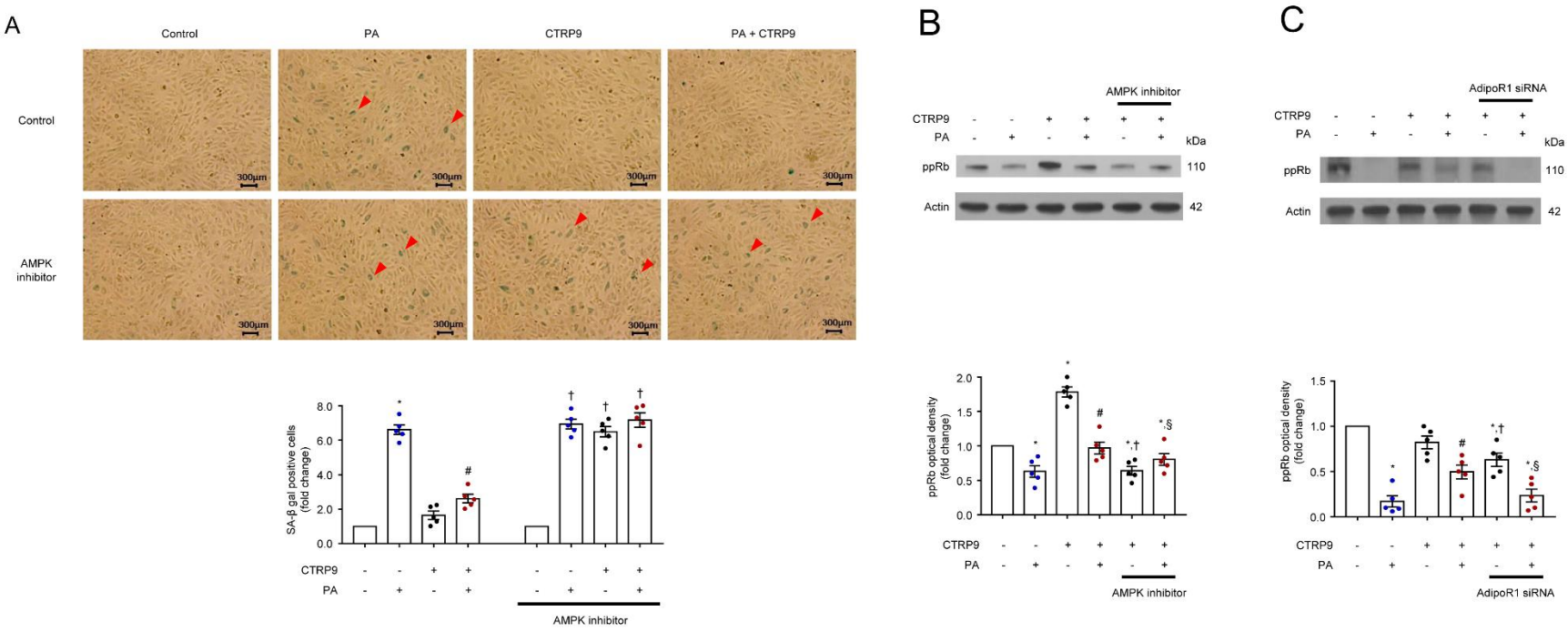


6. Inhibition of AMPK attenuates preventive effects of CTRP9 on PA-induced endothelial cell senescence

Finally, we tested whether the inhibitory effect of CTRP9 on PA-induced endothelial senescence required the activation of AMPK. After HUVECs were pre-treated with the AMPK inhibitor (compound C), the number of SA-β-Gal positive cells was decreased. The results showed that the preventive effect of CTRP9 disappeared (Figure 8A). Similarly, the increased expression of ppRB by CTRP9 treatment was also attenuated after AMPK inhibition (Figure 8B).

AdipoR1/AMPK dependent mechanism is known to be involved in the beneficial effects by CTRP9 on endothelium [35, 36]. To confirm the involvement of AdipoR1 in the inhibition of PA-induced endothelial cell senescence by CTRP9, HUVECs were transfected with siRNA for AdipoR1. The increased expression of ppRb by CTRP9 treatment was abolished in cells transfected with AdipoR1 siRNA (Figure 8C), suggesting the crucial role of AdipoR1 in this CTRP9's anti-senescent effect against PA.

Figure 8. AMPK and AdipoR1 activation are essential for the inhibition of PA-induced endothelial cell senescence by CTRP9. (A, B) HUVECs were pre-treated with AMPK inhibitor and treated with 3 μg/mL CTRP9 in the presence or absence of 500 μM PA. Endothelial cell senescence was evaluated by SA-β-Gal staining (A) and measurement of the protein level of ppRB (B). The SA-β gal positive cells were stained green, indicated by arrowheads (representative cells). (C) HUVECs were transfected with AdipoR1 siRNA. The protein level of ppRB were measured after treatment with 3 μg/mL CTRP9 in the presence or absence of 500 μM PA. Data are presented as the mean ± SEM of five independent experiments. ($^{*}P$ <0.05 vs. untreated cells; $^{\#}P$ <0.05 vs. cells treated with PA alone; $^{\dagger}P$ <0.05 vs. cells treated with AMPK inhibitor alone; $^{\S}P$ <0.05 vs. cells treated with CTRP9 and PA).


A
Control
PA
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CTRP9
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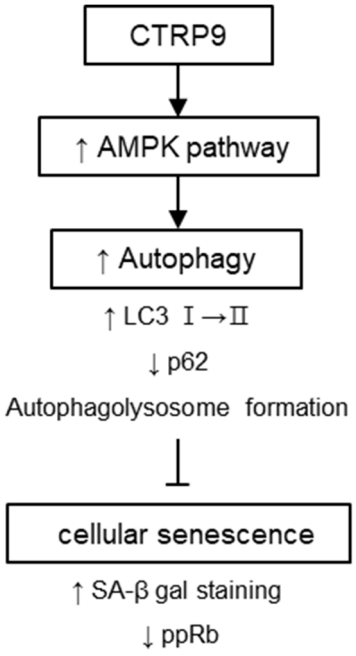
CTRP9
PA
kDa
ppRb
110
Actin
42
ppRb optical density (fold change)
C
AdipoR1 siRNA

Discussion

This study demonstrated that CTRP9 inhibited endothelial senescence induced by the PA FFA by promoting autophagy and autophagic flux. Activation of AMPK pathway was responsible for anti-senescent effect of CTRP9 due to the stimulation of autophagy and autophagic flux. CTRP9 has been suggested to act as regulator of vascular function, with beneficial effects on atherosclerosis and CVD [16, 37]. These findings were shown schematically in Figure 9.

Figure 9. A breif schematic dagram of anti-senescence mechanism of CTRP9 through autopahgy.



In several previous animal studies, CTRP9 contributed to an advantageous vascular effect, especially concerning atherosclerosis [35, 38-40]. CTRP9 was demonstrated to mediate endothelium-dependent vasorelaxation [35] and suppress vascular smooth muscle cell proliferation and neointimal formation [38]. Furthermore, elevated levels of CTRP9 improved the vascular plaque stability and alleviated the development of atherosclerosis in ApoE knockout mice by decreasing pro-inflammatory cytokines in macrophages [39, 40]. The current findings are consistent with previous results [35, 38-40] in terms of the protective effects of CTRP9 in the pathogenesis of atherosclerosis.

Adipokines are bioactive substances secreted by adipose tissue. These adipokines include adiponectin, leptin, omentin, and CTRPs [16]. CTRP9 has attracted a lot of attention since it was discovered in 2009 [41]. Accumulating evidence has indicated the protective effects of CTRP9 against atherosclerosis through multiple mechanisms including inhibition of the inflammatory response [17, 39, 40, 42, 43] and amelioration of endothelial dysfunction [16, 42, 44]. The findings implicate the CTRP9 adipokine as a promising target to prevent and treat atherosclerosis-associated disease. CTRP9 has also been reported to have beneficial effects on glucose metabolism, cellular survival, oxidative stress regulation, and inhibition of endoplasmic reticulum stress, all of which are associated with the aging mechanism of endothelial cells [45]. A previous study on the effect of CTRP9 on cellular senescence demonstrated that CTRP9 treatment prevented the oxidation reaction associated with aging in

mesenchymal stem cells [33]. Although the anti-aging role of CTRP9 has been of interest, the protective role of CTRP9 in FFA-induced endothelial senescence had not been fully investigated. We presently confirmed the preventive effect of CTRP9 on this process by checking the level of the senescence mediator ppRB (Figure 1B), as well as by SA-β-GAL staining (Figure 1A). Endothelial senescence is believed to be associated with atherosclerosis [1]. Thus, our current findings also support the anti-atherogenic effects of CTRP9.

Autophagy is an intracellular self-digestion mechanism that plays an important role in the degradation of cellular debris and exhausted organelles [20]. The process of autophagy is believed to mediate anti-aging effects [46]. Recent evidence has shown that the autophagic process is crucial in the pathogenesis of atherosclerosis [47]. However, whether autophagy reduces or promotes atherosclerosis is unclear. Autophagy maintains atherosclerotic plaques against oxidative stress by degrading damaged components and contributing to cellular retrieval [48]. Thereby, favorable autophagy can prohibit the apoptosis of macrophages and stabilize the atherosclerotic lesion [48]. In addition, the important role of autophagy in the control of apolipoprotein B (apoB) secretion from the liver was recently described. Accumulation of apoB in vessel walls is the inevitable initiating event in the progression of atherosclerosis [49]. Moreover, by diminishing intracellular damage, autophagy has been recognized as having an anti-aging function and has been shown to have protective effect in vascular endothelial senescence [50, 51]. However, excess activated autophagy or impaired

autophagy may lead apoptotic death of vascular smooth muscle cells, causing plaque instability [52].

Based on the previous results on the association of both CTRP9 and autophagy with cellular senescence and atherosclerosis [24, 40], we assessed whether autophagy allowed CTRP9 to prevent endothelial senescence. We demonstrated that CTRP9 reversed the suppression of autophagy that had resulted from PA treatment and recovered autophagic flux (Figure 5, 6). The inhibition of autophagy by transfection of LC3 siRNA supported an effect of autophagy on the anti-aging role of CTRP9 in vascular endothelial cells (Figure 2). Although the effect of autophagy in atherosclerosis was not fully investigated, our results suggest another crucial anti-atherogenic mechanism of CTRP9 through its anti-aging functions via autophagy in vascular endothelial cells.

Endothelial senescence attenuates the abilities of vascular repair, regeneration, and nitric oxide (NO) production [1]. In endothelial cells, senescence increases oxidative stress and expression of pro-inflammatory cytokines, mainly tumor necrosis factor-alpha [28] and nuclear factor-kappa B [53]. In addition, endothelial aging is induced by various factors including impaired mitochondrial function [54], elevated serum glucose [4], genetic factors [5], and lipotoxicity [6]. Among these factors that induce endothelial senescence, PA can cause lipotoxicity when present in excess in non-fat cells [28]. Elevated concentrations of FFA impair endothelial function through the increase in IKKβ activity, which is the main signaling

pathway responsible for cellular senescence, and are critical role in impairment of NO production in endothelial cells [7, 8]. Previous studies reported that PA induced ER stress, ROS, and apoptosis [23-26]. Endothelial senescence is known to be induced by increased production of ROS and ER stress [2, 6]. Thus, we used PA to induce cellular senescence of HUVECs in our experiments. We also observed that PA treatment affected cellular senescence (Figure 1).

Presently, exposure to PA decreased the autophagic flux (Figure 5A, lower) and inhibited the fusion of autophagosomes and lysosomes (Figure 6). However, conflicting findings have been reported regarding the function of FFAs in autophagy induction. In previous studies using pancreatic β-cells [55] and mouse embryonic fibroblasts [56], PA induced autophagy to protect cells from lipotoxicity. However, other reports including mouse fibroblasts [57] and human aortic endothelial cells [51] showed consistent findings with our results. PA exposure inhibited autophagosome–lysosome fusion, and thus alleviated autophagy [51, 57]. These conflicting results might be due to multiple factors such as different cell types, different degree of lipotoxicity, and study design [56]. Further studies are required to explain the heterogeneity in the lipotoxicity-induced autophagic induction in various conditions.

A number of signaling pathways are involved in regulating the initiation and maturation of autophagy [58]. One pathway includes mammalian target of rapamycin (mTOR), which acts as a gate-keeper in the inhibition of autophagy [58]. Inhibition of the mTOR pathway is related to anti-atherogenic effects and atherosclerotic plaque stabilization [59]. Besides mTOR

inhibitors, mitogen-activated protein kinase (MAPK) pathways including c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) are also play crucial roles in anti-atherogenic effects in autophagy [59]. Although we did not investigate mechanisms other than the AMPK pathway, previous studies have shown that AMPK activation is a positive regulator of autophagy by attenuating its downstream target of mTOR [60].

AMPK is an energy sensor that has various anti-atherogenic effects and is beneficial to endothelial function [61]. CTRP9 exerts its anti-atherogenic effects via the AMPK pathway, which include an anti-inflammatory effect in vascular endothelial cells [17, 47]. Based on the previous relationship between CTRP9 and the AMPK pathway, we evaluated whether the AMPK pathway was involved in autophagic induction by CTRP9. We observed that the inhibition of AMPK significantly reduced LC3 conversion induced by CTRP9 treatment (Figure 7A). In accordance with previous studies, the activation of AMPK has been demonstrated to be crucial in autophagic induction by CTRP9 [20, 24, 47]. When vascular endothelial cells were pre-treated with the AMPK inhibitor, significant decreases of the CTRP9-induced autophagy and autophagic flux were observed (Figure 7A, 7B). In addition, pre-treatment with the AMPK inhibitor also attenuated the protective role of CTRP9 on PA-induced endothelial cell senescence (Figure 8A, 8B). These findings suggest a mechanism in which AMPK activation mediates the effect of CTRP9 in preventing endothelial senescence via the induction of autophagy and autophagic flux. Further investigations will be needed to

determine whether other mechanisms might be involved in the autophagic induction by CTRP9.

This study has several limitations. First, as we used only HUVECs for examining anti-senescent effect of CTRP9 on endothelium, the generalizability of the results might be limited. Second, although CTRP9 is known as the closest adiponectin paralog, ~~however,~~ we did not used other adiponectin as a positive control to prove the anti-senescent effect of CTRP9. Therefore, we cannot mention its anti-senescent effect in a comparative manner. Lastly, as only PA was used to induce endothelial senescence in our study, further studies are needed to confirm whether CTRP9 might exert its anti-senescent effect in other metabolically deranged state.

Conclusion

CTRP9 can attenuate PA-induced endothelial senescence by recovering autophagy and autophagic flux. AMPK activation is involved in this protective effect of CTRP9 on cellular senescence. Further research using in vivo models will be needed to expand these findings and to identify CTRP9-based therapies for atherosclerosis.

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국문요약

배경: 자가포식은 죽상동맥경화의 병인에 중요한 과정으로 알려져 있다. C1q/tumor necrosis factor-related protein 9 (CTRP9)은 아디포넥틴 (adiponectin)과 가장 유사한 이원체 (paralog)로써 노화 방지 및 죽상동맥경화 방지 효과가 있다고 알려져 있다. 하지만 CTRP9 이 자가포식 및 혈관내피세포 노화에서 역할은 연구된 바가 없다. 따라서 본 연구에서 저자들은 CTRp9 이 자가포식 증가를 통해 혈관내피세포 노화에 미치는 영향을 알아보고자 하였다.

연구방법: 인간제대정맥내피세포 (Human umbilical vein endothelial cell; HUVEC)에 3 μg/mL 의 CTRP9 을 처치하고 이후 24 시간 동안 500 μM 팔미트산을 처치한 세포와 처치하지 않은 세포에서 노화의 정도를 평가하였다. 혈관내피세포 노화는 senescence associated acidic β-galactosidase 염색과 hyperphosphorylated retinoblastoma 단백질(pRB)로 측정하였다. 자가포식은 LC3 전환 비율과 자가포식을 통해 분해된 단백질인 p62/SQSTM1 로 평가하였다. 자가포식소체(autophagosome)-리소좀 융합은 형광현미경을 통해 확인하였다.

결과: CTRP9 을 처치하면 팔미트산으로 유발된 혈관내피세포 노화가 억제된다. CTRP9 은 LC3- 에서 LC3- 로 전환을 증가시켰으며 CTRP9 의 처치 시간과 용량 의존적으로 p62 수치를 감소시켰다. CTRP9 와 팔미트산 처치 모두 LC3 전환을 증가시켰지만, 팔미트산 처치는 p62 를 증가시키고 자가포식소체-리소좀 융합을

감소시켰으며 이는 감소된 자가포식 유동 (autophagic flux)을 나타낸다. 그러나 CTRP9 을 처치한 세포에서는 팔트미트산에 의해 억제된 자가포식 유동이 회복되었다. CTRP9 은 AMP-activated kinase (AMPK) 활성화를 통해 LC3 전환을 증가시키고 p62 를 감소시켰다.

결론: CTRP9 은 AMPK 활성화를 통해 자가포식 작용을 증가시켜 혈관내피세포 노화를 억제한다.

중심단어: C1q/TNF-related protein-9, 혈관내피세포 노화(endothelial senescence), 자가포식(autophagy), 죽상동맥경화증(atherosclerosis)