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Activation of autophagy during farnesyl pyrophosphate synthase inhibition is mediated through PI3K/AKT/mTOR signaling

Jie Han[1], Chaoyang Huang[1], Jiukun Jiang[2] and Dongmei Jiang[3]

Abstract

Objectives: Autophagy is divided into three phases: autophagosome engulfment of intracellular organelles and proteins, autophagosome fusion with lysosomes, and autolysosome degradation. The farnesyl pyrophosphate synthase inhibitor ibandronate (IBAN) has *in vivo* cardioprotective properties, potentially via anti-oxidant effects. Whether autophagy is involved in the cardioprotective effect of IBAN remains unexplored.

Methods: Human umbilical vein endothelial cells (HUVECs) were treated *in vitro* with IBAN to assess autophagy induction. Lysosomal activation and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling were assessed using a LysoTracker assay, acridine orange staining and western blotting. An MTS assay was used to assess cellular proliferation. Autophagy was inhibited using chloroquine or RNA silencing of autophagy-related 7 (Atg7) expression.

Results: IBAN induced autophagy in HUVECs. Moreover, IBAN activated lysosomal function, which is pivotal to autophagy induction. PI3K/AKT/mTOR activity was inhibited in IBAN-treated HUVECs, indicating the involvement of this pathway in IBAN-induced autophagy. Inhibition of autophagy using either chloroquine or Atg7 siRNA potentiated inhibition of HUVEC growth by

[1]Department of Cardiology, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China
[2]Department of Emergency, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China
[3]Department of Cardiology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China

Corresponding author:
Dongmei Jiang, Department of Cardiology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, 3 East Qingchun Road, Hangzhou, Zhejiang 310016, China.
Email: 3312024@zju.edu.cn

IBAN, suggesting the involvement of non-autophagy pathways in the antiproliferative effects of IBAN.

Conclusions: These findings provide insights into the role of autophagy in the cardioprotective effects of IBAN and the molecular mechanisms underlying autophagy induction by IBAN.

Keywords
Autophagy, HUVECs, FPPS, IBAN, mTOR, Atg7

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Introduction

Autophagy is an evolutionarily conserved mechanism through which cells can degrade and digest cellular components, providing an adaptive benefit under various stress conditions such as nutrient starvation.[1,2] The autophagy process can be divided into several organized phases: (i) engulfment of cytoplasmic components, such as organelles, by autophagosomes; (ii) formation of autolysosomes by fusion with lysosomes; and (iii) proteolytic degradation and recycling of basic materials.[3] The main role of autophagy is to degrade and recycle housekeeping proteins and damaged cellular apparatus under nutrient-rich conditions. During robust cellular growth, levels of autophagy are minimal. In contrast, in the presence of stressful stimuli such as nutrient deprivation, autophagy is induced. Autophagy has been proposed to be involved in multiple physiological processes including cellular development, innate immunity, cell survival, and cell death. Pathological conditions in which autophagy may play a role include malignancy, degenerative neurological conditions, and metabolic disorders.[4,5]

Bisphosphonates inhibit farnesyl pyrophosphate (FPP) synthase (FPPS), an enzyme which plays a pivotal role in the mevalonate pathway.[6] FPPS is involved in synthesis of various isoprenoids, including FPP and geranylgeranyl pyrophosphate (GGPP), and consequently can inhibit isoprenylation processes such as farnesylation and geranylgeranylation.[7,8] GGPP is indispensable for isoprenylation and activation of Rac1.[9,10] Wasko et al.[11] found that bisphosphonates could inhibit FPPS and geranylgeranyl diphosphate synthase (GGDPS) leading to autophagy by depleting GGPP. Moreover, zoledronic acid, a third-generation nitrogen-containing bisphosphonate that acts primarily through FPPS and geranylgeranyl pyrophosphate synthase (GGPPS),[12,13] was found to induce autophagy via oxidative stress. The effects of zoledronic acid could be prevented by treatment with anti-oxidants.[14] However, the roles of autophagy in regulating cardiovascular endothelial cell oxidative injury and repair have not yet been fully investigated.

In our previous study, the FPPS inhibitor ibandronate (IBAN) was shown to have anti-oxidant effects in cultured vascular smooth muscle cells (VSMCs) and in the vasculature of spontaneously hypertensive rats (SHRs). These effects were mediated via the Rac1/nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathway.[15] Whether IBAN could induce autophagy in endothelial cells and whether the cardioprotective effects of IBAN were related to autophagy induction have yet to be determined. Here, we showed that IBAN induced autophagy in human umbilical vein endothelial cells (HUVECs) via inhibition of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target

of rapamycin (mTOR) pathway in a time- and concentration-dependent manner. Moreover, blocking autophagy potentiated IBAN-induced inhibition of endothelial cell growth.

Materials and methods

Reagents and cell culture

HUVECs were cultured in six-well plates until they reached confluence ($1–5 \times 10^5$ cells per mL). Sodium IBAN and chloroquine (CQ) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HUVECs were cultured in Dulbecco's modified eagle medium (Thermo Fisher Scientific, Shanghai, China) supplemented with 10% fetal bovine serum (Biological Industries Israel Beit Haemek Ltd., Israel), 100 U/mL penicillin and 100 U/mL streptomycin (Genom, Hangzhou, China).

Autophagy assays

HUVECs were transfected with tandem fluorescently-tagged microtubule-associated protein 1 light chain 3 (LC3) [monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP)-LC3] constructs (Hanbio, Shanghai, China) using Lipofectamine 2000 (Thermo Fisher Scientific) to monitor autolysosome and autophagosome formation. Red and green fluorescent puncta were quantitated by confocal microscopy. The autophagosome marker protein LC3 and the autophagy flux marker protein p62 were detected by western blotting using anti-LC3 (Cell Signaling Technology, Shanghai, China) and anti-p62 (Abcam, Shanghai, China) antibodies.

Lysosomal assays

LysoTracker (Thermo Fisher Scientific) is a red fluorescent probe that is selectively retained in acidic lysosomes to achieve specific fluorescent labelling of lysosomes. High lysosomal integrity and lower pH will produce a vigorous fluorescence intensity. Acridine orange (AO) (Thermo Fisher Scientific) staining can also be used to identify acidic lysosomes. Activated lysosomes show higher cathepsin B enzyme activity, and Cathepsin Magic Red™ (Bio-Rad, Hercules, CA, USA) was used to confirm changes in lysosomal activity. Image data were analyzed using ImageJ software (ImageJ 1.4.3.67, National Institutes of Health, Bethesda, MD, USA).

Western blotting and mTOR signaling assays

Total protein was extracted from HUVECs using lysis buffer (Thermo Fisher Scientific). A bicinchoninic acid protein assay kit (Beyotime, Shanghai, China) was used to quantify protein content. Equivalent amounts of total protein for each sample were separated on SDS-PAGE gels (Multi Sciences, Hangzhou, China) and transferred to polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). The membranes were blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% skim milk. Primary anti-LC3, anti-p62, anti-phospho-PI3K, anti-PI3K, anti-phospho-AKT, anti-AKT, anti-phosphorylated 70-kDa ribosomal S6 kinase (phospho-p70S6K), anti-p70S6K, anti-phospho-S6, anti-S6, and anti-α-tubulin antibodies were obtained from Abcam. α-tubulin was used as a loading control. After washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG secondary antibody (Multi Sciences). Bands were visualized using the BeyoECL Star kit (Beyotime). Image data were analyzed using Image J software.

Cell proliferation assays

HUVEC proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay (BioVision, Inc., Milpitas, CA, USA). The cells were plated in 96-well plates at a density of 4,000 cells/well. The experimental procedure and data analysis were conducted according to the manufacturer's instructions. The absorbance was measured at 490 nm using an Epoch 2 microplate reader (BioTek, Winooski, VT, USA).

RNA silencing of autophagy-related 7 (Atg7) expression

The siRNA for Atg7 was obtained from Cell Signaling Technology (SignalSilence® Atg7 siRNA I #6604). HUVECs were transfected with the specific siRNA using Lipofectamine 2000 (Thermo Fisher Scientific). Western blotting was performed to assess Atg7 knockdown. Primary antibodies against Atg7 were from Abcam. α-tubulin was used as the loading control.

Statistical analysis

Continuous data were expressed as means ± standard deviations. Differences among groups were assessed using one-way analysis of variance followed by Fisher's least significant difference *post-hoc* test. Values of $P < 0.05$ were considered statistically significant.

Results

FPPS inhibition induced autophagy in HUVECs

HUVECs treated with the FPPS inhibitor IBAN were co-transfected with expression vectors encoding RFP-LC3 and GFP-LC3. Following transfection, red dots represented autolysosomes and yellow dots represented autophagosomes. Confocal microscopy showed that IBAN increased the number autolysosomes and promoted autophagy (Figure 1a; $P<0.001$ versus control group). HUVECs were treated with various concentrations of IBAN (Figure 1b: 25, 50 and 100 μM) for different durations (Figure 1c: 6, 12 or 24 hours). Western blotting showed that IBAN treatment increased LC3-II expression (an autophagosome marker) in a time- (Figure 1c and e) and concentration-dependent manner (Figure 1b and d).[16] Autophagy flux was also assess via decreases in protein levels of p62, which is a well-recognized substrate during autophagy (Figure 1b,f,c and g).

FPPS inhibition activated the lysosomal function of HUVECs

Several methods were used to assess lysosomal activity in HUVECs. LysoTracker staining showed increased fluorescence intensity in HUVECs following IBAN treatment, suggesting enhanced acidification of lysosomes (Figure 2a). This finding was further supported by enhancement of red color intensity following staining with AO (Figure 2b). AO is a chelating dye which shows orange/red fluorescence, and its accumulation indicated increased acidity in the lysosome.[17] Activity of the lysosomal enzyme cathepsin B was subsequently assessed using a Cathepsin Magic Red™ kit. After 12 hours of IBAN treatment in HUVECs, we observed a two-fold increase in fluorescence intensity, reflecting increased cathepsin B activity (Figure 2c).

Autophagy and lysosomal activation by an FPPS inhibitor occur via suppression of PI3K/AKT/mTOR signaling

PI3K/AKT/mTOR signaling is a major molecular mechanism mediating lysosomal function during autophagy.[18,19] IBAN

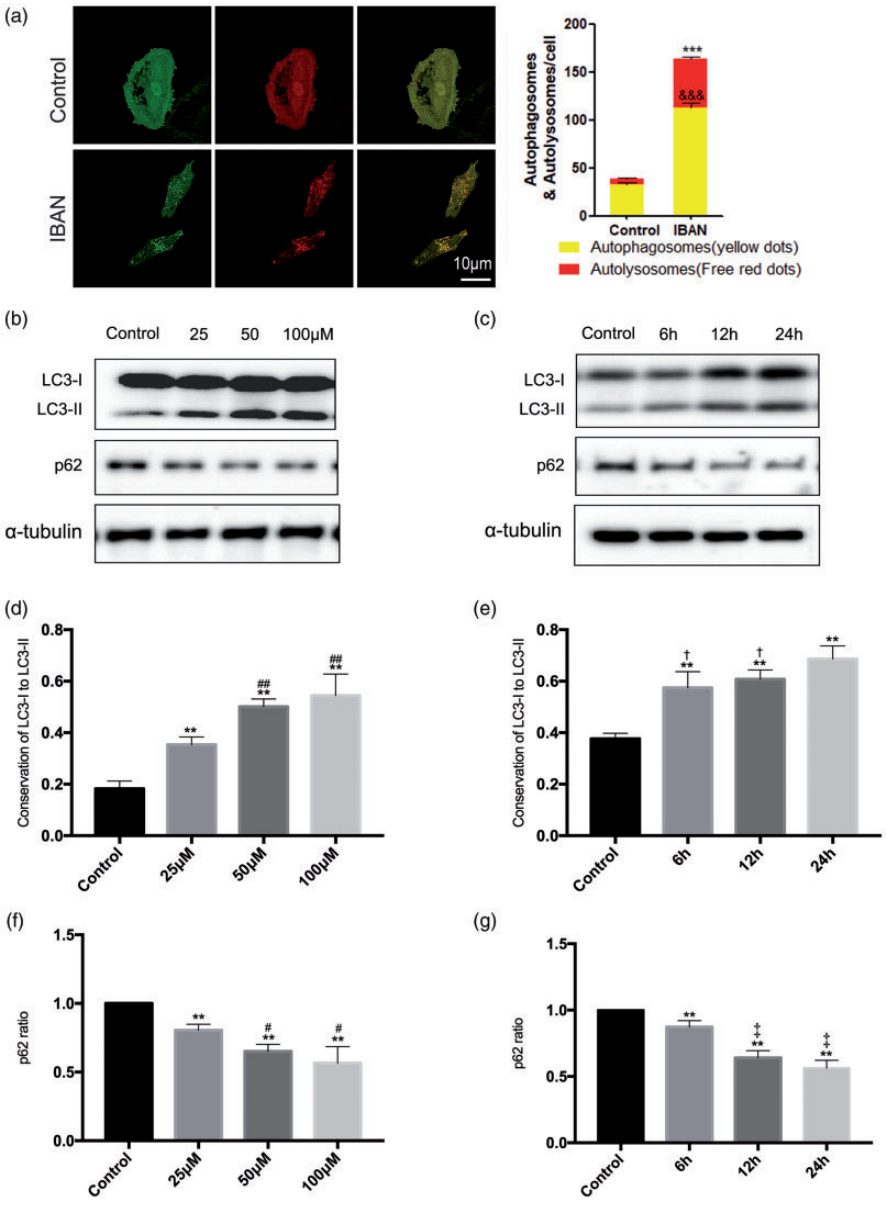

Figure 1. FPPS inhibition induced autophagy in HUVECs. (a) HUVECs treated with the FPPS inhibitor IBAN (100 μM, 12 hours) were co-transfected with RFP-LC3 and GFP-LC3 and observed under confocal microscopy (scale bar: 10 μm). Red dots represent autolysosomes and yellow dots represent autophagosomes. $^{***}P < 0.001$ and $^{\&\&\&}P < 0.001$ versus control group. (b) HUVECs were treated with different concentrations of IBAN for 24 hours. Western blotting was used to assess LC3-I, LC3-II and p62 expression. Histograms were used to display the results of western blotting analysis for LC3 (d) and p62 (f). (c) HUVECs were treated with IBAN (100 μM) for different time periods as indicated. Western blotting was used to detect LC3-I, LC3-II and p62 expression. Histograms were used to display the results of western blotting analysis for LC3 (e) and p62 (g). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus 25 μM group, $^{\dagger}P < 0.05$ versus 24 hour group and $^{\ddagger}P < 0.01$ versus 6 hour group.

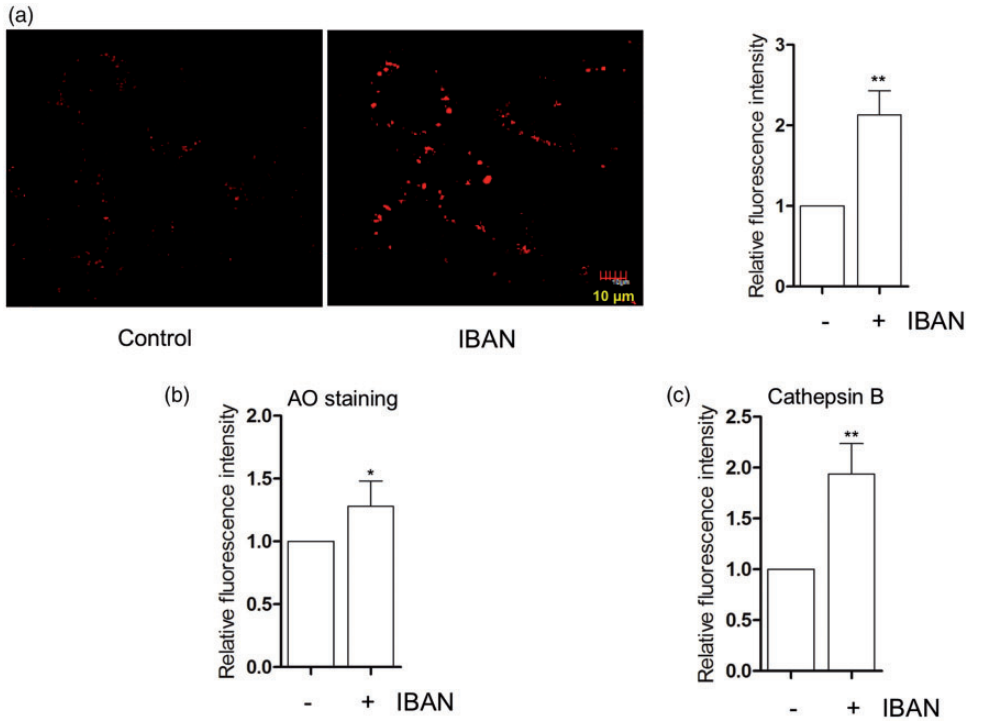

Figure 2. FPPS inhibition activates lysosomal function. (a) HUVECs were treated with IBAN (100 μM) for 12 hours and then stained with LysoTracker Red DND-99 (50 nM) for 15 minutes. Fluorescence intensity of treated cells was measured using confocal microscopy (left) or flow cytometry (right). Scale bar, 10 μm. (b) and (c) As indicated in (a), after treatment with IBAN for 12 hours, cells were stained with acridine orange staining and Magic Red (cathepsin B) and analyzed using flow cytometry. *$P < 0.05$ and **$P < 0.01$ versus control group.

treatment decreased phospho-PI3K, phospho-AKT, phospho-p70S6K and phospho-S6 levels in HUVECs in a time- and concentration-dependent manner (Figure 3). The inhibition of these proteins suggested that the PI3K/AKT/mTOR pathway was suppressed. These results suggested that FPPS inhibition induced autophagy and lysosomal activation through suppression of PI3K/AKT/mTOR signaling.

Potentiation of cell growth inhibition following inhibition of autophagy induction

The functional role of autophagy activation after IBAN treatment was assessed by analyzing its effect on cell growth. CQ can be used as a pharmacological inhibitor of lysosome formation because it can raise the lysosomal pH and inhibit autophagy by blocking autophagosome-lysosome fusion and lysosomal protein degradation.[20] As shown in Figure 4a, morphological changes indicated that IBAN inhibited cell growth more extensively in the presence of CQ. Next, the MTS assay confirmed that CQ potentiated IBAN inhibition of HUVEC proliferation (Figure 4c). Silencing of Atg7 expression in HUVECs, which was confirmed by western blotting (Figure 4e), also potentiated the inhibitory effect of IBAN on HUVEC proliferation

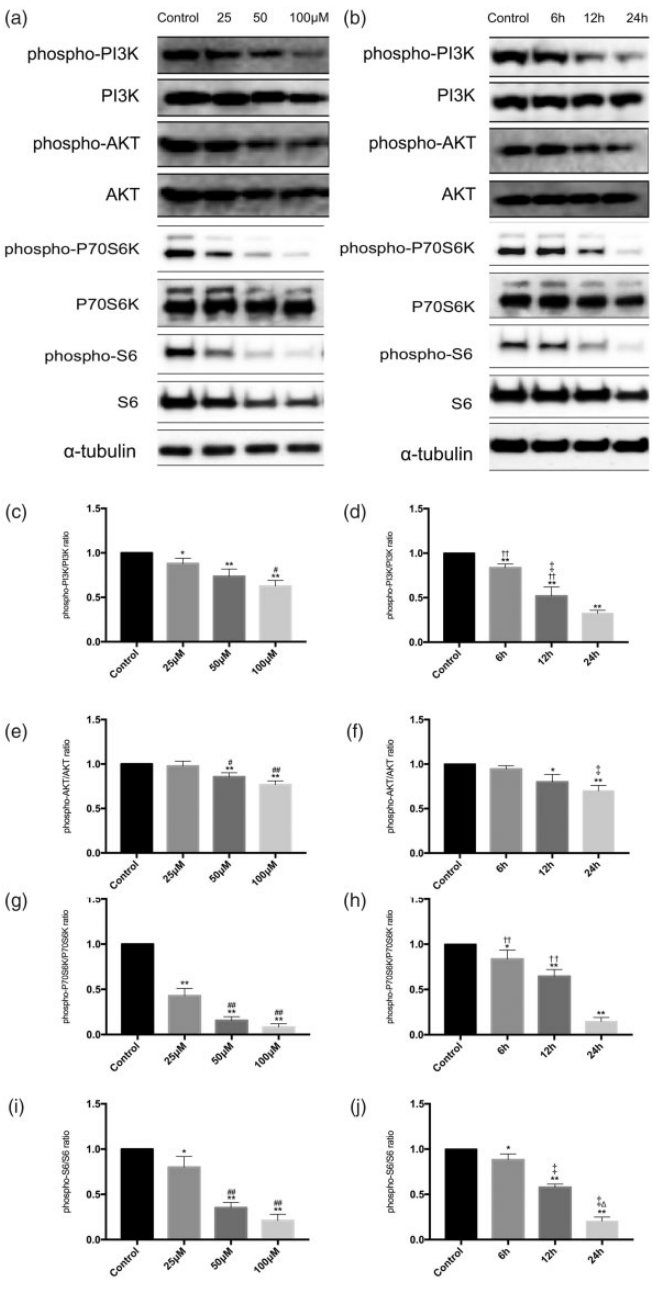

Figure 3. FPPS inhibition suppresses PI3K/AKT/mTOR signalling in HUVECs. (a) HUVECs were treated with different doses of IBAN for 24 hours. Representative western blotting images are shown for phospho-PI3K, PI3K, phospho-AKT, AKT, phospho-p70S6K, p70S6K, phospho-S6 and S6. Bands were quantified via densitometry and normalized to α-tubulin. The ratios between phosphorylated proteins and total proteins are shown in c, e, g and i respectively. (b) HUVECs were treated with IBAN (100 μM) for different time periods as indicated. Cells were collected and lysed, and cell lysates were prepared for

(Figure 4b,d). These observations indicated a milder inhibitory effect of IBAN on cellular proliferation via induction of autophagy in HUVECs. Thus, there may have been additional pathways through which cellular growth was impacted, potentially due to either direct or indirect inhibition of PI3K/AKT/mTOR signaling by IBAN.

Discussion

Autophagy exerts a protective role against injury in HUVECs, including against damage caused by advanced glycation end-products[21] and oxidative stress.[22] Our previous study showed that inhibition of FPPS improved endothelial function in SHRs, at least in part via the Rac1/NADPH oxidase pathway.[15] Moreover, IBAN leads to decreased production of reactive oxygen species and decreased expression of NADPH oxidase subunits in cultured VSMCs and SHR aortas. In our current study, we confirmed that the FPPS inhibitor IBAN inhibited PI3K/AKT/mTOR activity and autophagy induction in HUVECs. Induction of autophagy in HUVECs actually represented a milder insult to cell proliferation.

The isoprenoid biosynthetic pathway is involved in generation of a range of molecules with different biological activities. Inhibition of the isoprenoid biosynthetic pathway has been linked to autophagy, but the precise mechanisms through which isoprenoid biosynthesis inhibitors induce autophagy are not yet known. Wasko et al.[11] reported that inhibition of FPPS and GGDPS by bisphosphonates such as zoledronate and digeranyl bisphosphonate respectively induced autophagy by depleting cellular GGPP and interfering with protein geranylgeranylation. Moreover, Khandelwal et al.[14] demonstrated that zoledronic acid, an inhibitor of FPPS and GGPPS, induced autophagy via oxidative stress and that anti-oxidants inhibited zoledronic acid-induced autophagy. Our current work on the FPPS inhibitor IBAN also confirmed its ability to induce autophagy in HUVECs, which was consistent with previous reports. In addition, activation of lysosomal function was observed during the late stages of autophagy, indicating its involvement in IBAN-initiated autophagy induction.

Nutrient starvation has been widely used to elicit autophagy. Nutrient stress typically results in activation of the AMP-activated protein kinase (AMPK) and inactivation of the mTOR complex 1 (MTORC1) pathways.[23] AMPK and MTORC1 signals converge by phosphorylation and regulation of the unc-51-like kinase1(ULK1).[24] ULK1 or unc-51-like kinase2 (ULK2) are pivotal players in the unc-51-like kinase1/2 (ULK1/2) autophagy regulatory complex, which can incorporate additional molecular factors including ATG13, ATG101, and FIP200. Once activated, the ULK1/2 complex has been shown to initiate formation of autophagosome and to increase cellular autophagy levels.[1] In our study, IBAN inhibited PI3K/AKT/mTOR activity as shown via reduced levels of phospho-p70S6k) and phospho-S6 levels, suggesting that PI3K/AKT/mTOR was a downstream pathway mediating HUVEC autophagy

Figure 3. Continued.
western blotting. α-tubulin was used as a loading control. Representative western blotting images are shown for phospho-PI3K, PI3K, phospho-AKT, AKT, phospho-p70S6K, p70S6K, phospho-S6 and S6. Bands were quantified via densitometry and normalized to α-tubulin. The ratios between phosphorylated proteins and total proteins are shown in d, f, h and j, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus 25 μM group, $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$versus 24 hour group, $^{\ddagger}P < 0.01$ versus 6 hour group and $^{\Delta}P < 0.01$ versus 12 hour group.

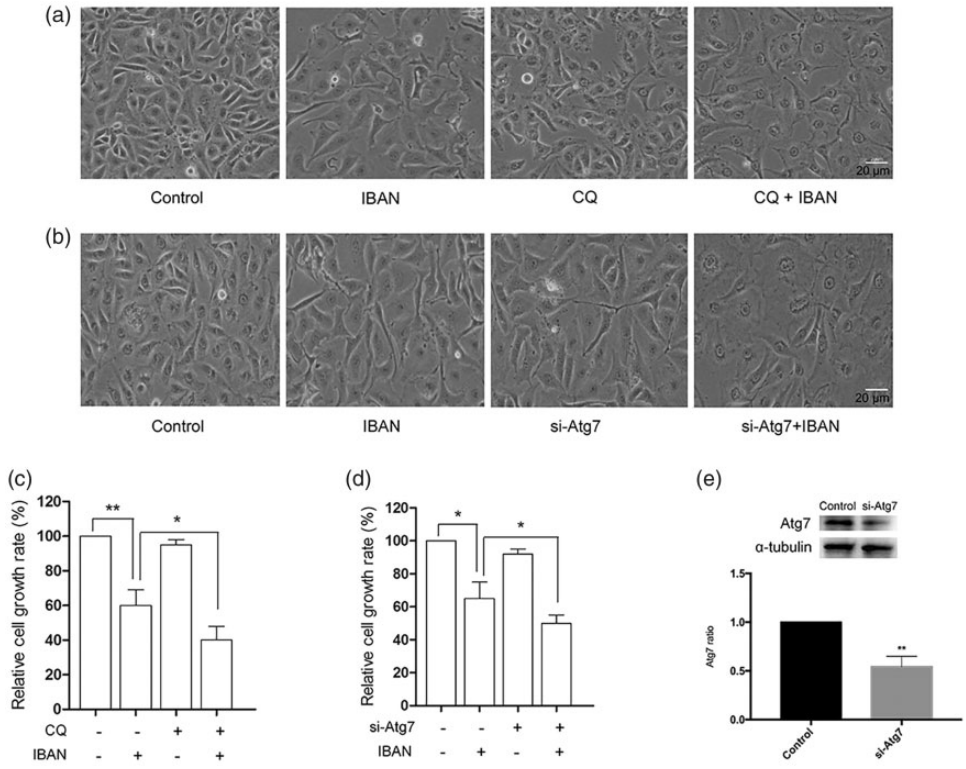

Figure 4. FPPS inhibition potentiates cell growth inhibition via impairment of autophagy. HUVECs were treated with IBAN (100 μM) with or without chloroquine (25 μM) for 24 hours. (a,b) Morphological changes of HUVECs following treatments were visualized using an inverted microscope (scale bar: 20 μm). (c) Cell proliferation was assessed using an MTS assay. The formazan dye produced by viable cells was quantified by measuring absorbance at 490 nm. (d) HUVECs were transiently transfected with an Atg7-specific siRNA. HUVECs were subsequently treated with IBAN (100 μM) for 24 hours, and cell proliferation was assessed using an MTS assay. *$P < 0.05$, **$P < 0.01$. (e) Western blotting was used to confirm Atg7 knockdown. Histograms were used to display the results of western blotting analysis. **$P < 0.01$ versus control group.

induced by IBAN. Interestingly, induction of autophagy by IBAN decreased HUVEC growth, and blocking autophagy significantly potentiated this inhibitory effect. These exciting results suggested the existence of an alternative pathway mediating the apparent growth-limiting effect of IBAN on HUVECs. Induction of autophagy was relatively advantageous in terms of a milder restriction on endothelial cell growth; this finding was consistent with previous research showing that IBAN exerts an anti-proliferative effect on cells through apoptosis.[25] Therefore, our results provide novel insights into the *in vitro* biological role of IBAN in normal endothelial cell growth. These effects might help to explain the protective role of IBAN observed *in vivo*, including in SHRs. Further studies are needed to explore these additional non-autophagy pathways and to understand their interplay and involvement during various cardiovascular conditions.

In conclusion, our findings revealed a novel role for FPPS inhibition in induction of autophagy in endothelial cells. The mechanism of FPPS inhibition of autophagy involved inhibition of PI3K/AKT/mTOR signaling. Induction of autophagy might be involved in the cardioprotective and anti-oxidative stress effects of IBAN.

Author contributions

H.J. and C.H designed the study; H.J. performed most of the experiments and analyzed the data; H.C. and J.J. helped to perform the experiments and provided technical assistance; H.J. and J.D. wrote the manuscript; and J.D. supervised the study and provided critical revision of the manuscript.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

Ethics statement

This study did not involve animal or human subjects, and thus, ethical permission from the relevant Institutional Review Boards was not applicable.

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ORCID iD

Dongmei Jiang https://orcid.org/0000-0002-7926-8645

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