

# Impaired lipophagy in endothelial cells with prolonged exposure to oxidized low-density lipoprotein

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Received October 30, 2019; Accepted May 27, 2020

DOI: 10.3892/mmr.2020.11345

**Abstract.** Oxidative stress induces the formation of oxidized low-density lipoprotein (ox-LDL), which accelerates the development of atherosclerosis and the rupture of atherosclerotic plaques by promoting lipid accumulation and inhibiting autophagy in vascular cells. Lipophagy is known to be involved in maintaining the balance of neutral lipid metabolism; however, the phenomenon of lipophagy deficiency in ox-LDL-treated endothelial cells (ECs) remains to be elucidated. It has been demonstrated that lipid accumulation caused by ox-LDL inhibits autophagy, which promotes apoptosis in ECs. The aim of the present study was to investigate the association between decreased autophagy and lipid accumulation in ECs treated with ox-LDL. Electron microscopy demonstrated that the formation of autolipophagosomes was decreased in ox-LDL-treated human umbilical vein ECs compared with that in the LDL-treated group and was accompanied by a decrease in the autophagy-associated proteins via western blotting analysis. Using laser focal colocalization detection, decreased lipid processing was observed in the lysosomes of ox-LDL-treated ECs, which indicated that lipophagy may be

attenuated and subsequently result in lipid accumulation in ox-LDL-treated ECs.

## Introduction

Lipid accumulation in the arterial walls is one of the hallmarks of atherosclerosis (AS) (1), which is an important pathological manifestation of cardiovascular disease and a notable cause of mortality in numerous countries, such as the UK, Australia and USA (2). It has been demonstrated that monolayer endothelial cell (EC) dysfunction on the inner wall of arterial vessels is the initial step in the process of atherosclerotic plaque formation (3,4). EC dysfunction can be caused by oxidized low-density lipoprotein (ox-LDL), which is often induced by oxidative stress that can be caused by reactive oxygen species, smoking, diabetes and obesity (5-8). Under normal conditions, ECs can export hydrolysed LDL-derived cholesterol into extracellular high-density lipoprotein (HDL), but cannot efficiently efflux ox-LDL-derived cholesterol, resulting in lipid accumulation in ECs (9). Furthermore, the accumulation of ox-LDL in the endothelium has been significantly associated with the dysfunction and apoptosis of ECs (10,11).

Autophagy is a process of evolutionarily conserved catabolism, which degrades a number of cellular components, including long-lived proteins and various organelle components (12,13). A high LC3-II to LC3-I ratio reflects increasing autophagosome formation, whereas low p62 protein levels are associated with high autophagy flux (14,15). LAMP1 is an essential component of the lysosomal membrane, serving as a lysosomal biomarker with a key role in autophagy and lysosomal fusion (16). It has been demonstrated that autophagy can protect ECs from oxidative stress-induced cell damage (17). Since ox-LDL has been reported to be involved in the development of AS and the rupture of atherosclerotic plaques by inhibiting autophagy (18), it was hypothesized that endothelial autophagy serves a key role in limiting the accumulation of ox-LDL-associated lipids within vessel walls (19). An association between ox-LDL-associated autophagy and endothelial lipid accumulation has been identified; however, the mechanism remains unclear.

The specific degradation of lipids by autophagy (also known as lipophagy) was first described in the liver, where

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**Abbreviations:** ALPs, autolipophagosomes; AS, atherosclerosis; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; 7KC, 7-keto cholesterol; LAMP1, lysosomal-associated membrane protein 1; LDs, lipid droplets; ox-LDL, oxidized low-density lipoprotein; TEM, transmission electron microscopy

**Key words:** oxidized low-density lipoprotein, lipophagy, endothelial dysfunction, oxidative stress injury

genetic defects (mutations in RNAi-ATG5 and ATG7) of macro-autophagy led to the accumulation of lipid droplets (LDs) (20). Lipophagy serves a critical role in maintaining overall lipid homeostasis (21). It has been demonstrated that mice bearing autophagy-related 5-deficient macrophages exhibit increased plaque formation due to an impairment of lipophagy (22,23). Therefore, the present study hypothesized that the decrease in endothelial autophagy induced by ox-LDL may be due to impaired lipophagy, resulting in intracellular lipid accumulation and EC damage.

The current study investigated the association between autophagy and lipid accumulation in human umbilical vein endothelial cells (HUVECs) treated with ox-LDL. The results provide evidence that lipophagy may be attenuated in HUVECs with prolonged exposure to ox-LDL and may provide novel insights into the prevention and treatment of atherosclerotic cardiovascular disease.

## Materials and methods

**Cell culture and treatments.** HUVECs were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences and were maintained at 37°C with 5% CO<sub>2</sub> in high glucose DMEM supplemented with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin. HUVECs were treated with PBS (basal), LDL (50 µg/ml; control) or ox-LDL (100 µg/ml) for 6, 12, 24 and 48 h.

**Cell viability assay.** An MTT assay was used to determine the viability of HUVECs. Cells were seeded into 96-well microplates at a density of 4x10<sup>4</sup> cells/well and cultured 37°C for 24 h. Subsequently, HUVECs were treated with different concentrations of ox-LDL (25, 50 or 100 µg/ml) for 6, 12, 24 and 48 h, and 20 µl MTT solution (5 mg/ml; Beyotime Institute of Biotechnology) was added to each well. The plates were incubated at 37°C for 4 h and then DMSO was added to dissolve the purple formazan products. The optical density value at 490 nm was measured using a microplate reader spectrophotometer (Molecular Devices, LLC).

**Transmission electron microscopy (TEM).** After removing the culture medium, the HUVECs were washed twice with ice-cold PBS and fixed with 2.5% glutaraldehyde overnight at 4°C. The cells were post-fixed in 2% osmium tetroxide at 4°C for 1 h. Dehydration was subsequently performed in 50-90% ethanol, and the samples were embedded in epoxy resin at 45°C for 12 h. Ultrathin sections (70-nm) were cut using an ultramicrotome (Leica Microsystems GmbH) and counterstained with uranyl acetate for 10 min and lead citrate for 30 min at RT. The sections were examined using a Jeol JEM SX 100 electron microscope (magnification, x1,200) (JEOL, Ltd.).

**Oil Red O staining.** The HUVECs were seeded into 6-well plates (2.5x10<sup>5</sup> cells/well) and were treated as aforementioned. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Subsequently, the HUVECs were stained with a 0.3% Oil Red O staining solution for 8 min at room temperature and washed with 60% isopropanol for 5 sec. The cells were stained with

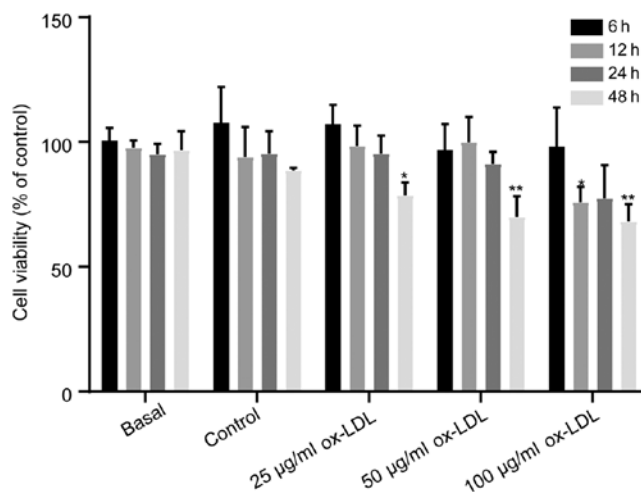


Figure 1. HUVEC viability is decreased by ox-LDL. HUVECs were treated with either PBS (basal group), 50 µg/ml LDL (control group) or 25, 50 or 100 µg/ml ox-LDL for 6, 12, 24 or 48 h. \*P<0.05; \*\*P<0.01 vs. control group (n=3). HUVEC, human umbilical vein endothelial cell; ox-LDL, oxidized low-density lipoprotein.

haematoxylin (Sigma-Aldrich; Merck KGaA) for 10 sec at RT prior to obtaining images using a light microscope (magnification, x40) (Nikon Corporation).

**Western blot analysis.** Total protein was extracted from HUVECs using a lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, leupeptin and EDTA) and was quantified using a bicinchoninic acid kit (Beijing ComWin Biotech Co., Ltd.). Equal amounts of proteins (30 µg/lane) were separated using 8-12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore). Following blocking with 5% BSA (Sigma-Aldrich; Merck KGaA) for 1.5 h at room temperature, the membranes were incubated with the following primary antibodies (all 1:1,000) at 4°C overnight: Anti-LC3B (cat. no. 3868S; Cell Signaling Technology, Inc.), anti-p62 (cat. no. ab91526; Abcam), anti-lysosomal-associated membrane protein 1 (LAMP1; cat. no. ab24170; Abcam) and anti-β-actin (cat. no. 4970S; Cell Signaling Technology, Inc.). Subsequently, the membranes were incubated with an anti-rabbit IgG HRP-linked secondary antibody (1:5,000; cat. no. 7074; Cell Signalling Technology) for 45 min at room temperature. The bands were visualized using an enhanced chemiluminescence reagent (EMD Millipore). The densitometry of each band was analysed using the Sigma Scan Pro5 software (version 5.0; Systat Software, Inc.) and normalized to β-actin values.

**Immunofluorescence staining.** The HUVECs were fixed with 4% paraformaldehyde (pH 7.4) for 20 min at RT, washed three times with PBS, blocked with 5% BSA at RT for 2 h, and then incubated with the following rabbit primary antibodies (all 1:200) overnight at 4°C: Anti-LAMP 1 (cat. no. ab24170; Abcam) and anti-LC3B (cat. no. 3868S; Cell Signaling Technology, Inc.). Subsequently, the cells were incubated with anti-rabbit IgG (H+L), F(ab')<sub>2</sub> Fragment (Alexa Fluor® 647 Conjugate) (red) antibodies (1:500; cat. no. 4414; Cell

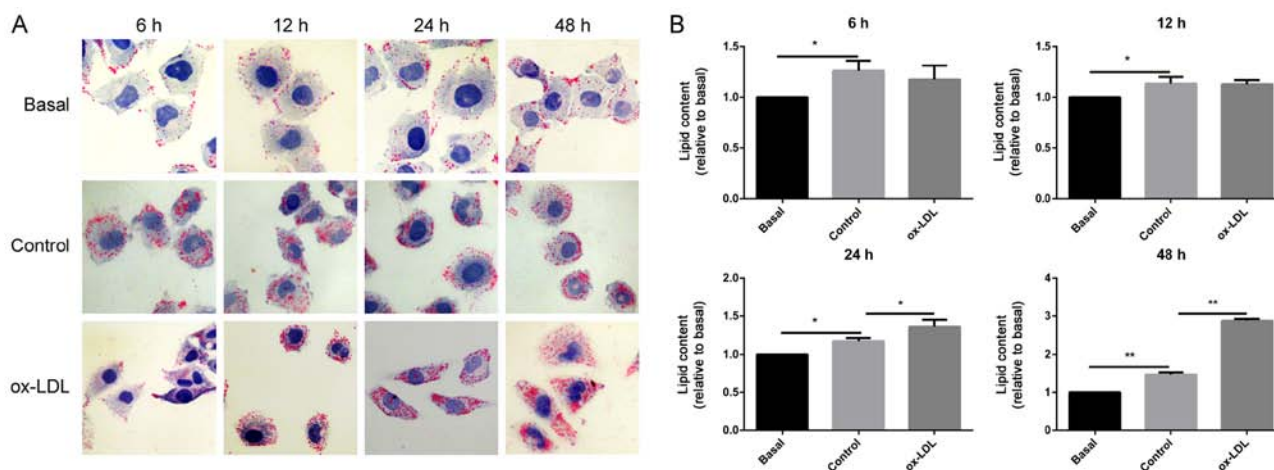


Figure 2. Neutral lipid accumulation of the HUVECs due to ox-LDL. HUVECs were treated with either PBS (basal group), 50  $\mu\text{g/ml}$  LDL (control group) or 100  $\mu\text{g/ml}$  ox-LDL for 6, 12, 24 or 48 h. (A) Lipid accumulation was detected using Oil Red O staining. (B) Quantification of neutral lipids was analysed using Fiji software. Magnification,  $\times 40$ . \* $P < 0.05$ ; \*\* $P < 0.01$  ( $n = 3$ ). HUVEC, human umbilical vein endothelial cell; ox-LDL, oxidized low-density lipoprotein.

Signaling Technology) for 1 h at 37°C. LDs were stained with 1  $\mu\text{g/ml}$  Bodipy 493/503 (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at RT in the dark, and with 10 mg/ml DAPI (cat. no. H-1200; Vector Laboratories, Inc.) at RT for 5 min. The cells were examined under a confocal laser scanning microscope (magnification,  $\times 400$ ) (LSCM 510 Meta; Zeiss AG). The relative colocalization coefficient between LC3, LAMP1 and LD was analysed via ImageJ (version no. 1.52P) software (24).

**Statistical analysis.** All values were presented as the mean  $\pm$  SD ( $n \geq 3$ ). The differences between groups were analysed via one-way ANOVA and post hoc Dunnett's multiple comparisons test by GraphPad Prism (version 6; GraphPad Software, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Lipid accumulation in HUVECs induced by ox-LDL.** To determine the appropriate concentration of ox-LDL, as an oxidative stress factor on ECs, the HUVECs were treated with different concentrations of ox-LDL (25, 50 or 100  $\mu\text{g/ml}$ ) for 6, 12, 24 and 48 h, and the cell viability was detected using the MTT assay. The results demonstrated that there was no difference in cell viability in HUVECs treated with 25  $\mu\text{g/ml}$  ox-LDL for 6, 12, and 24 h, whereas compared with corresponding control group, 50  $\mu\text{g/ml}$  ox-LDL for 48 h inhibited cell growth (Fig. 1). Furthermore, when treated with 100  $\mu\text{g/ml}$  ox-LDL, the cell viability significantly decreased at 12 and 48 h compared with the control group (Fig. 1), which is consistent with the finding that 100  $\mu\text{g/ml}$  ox-LDL decreased HUVEC viability when investigating the effect of ox-LDL on autophagy (25). Therefore, 100  $\mu\text{g/ml}$  ox-LDL was chosen as the oxidative stress factor for all subsequent experiments.

To investigate the deposited lipid effect on the ECs under oxidative stress, the LDs were stained with Oil Red O in HUVECs that had been exposed to 100  $\mu\text{g/ml}$  ox-LDL. As shown in Fig. 2, the results demonstrated that compared with the basal group, lipid droplets in HUVECs treated with LDL for

6, 12, 24 and 48 h were significantly increased, which indicates that HUVECs were lipid-loaded. Lipid staining in HUVECs showed a decreased tendency without statistical difference following treatment with ox-LDL for 6 h and 12 h compared with that in cells treated with LDL (control). However, when the HUVECs were treated with ox-LDL for 24 and 48 h, lipid deposition in the cells was significantly increased compared with that in the control group. The present data indicated that prolonged exposure to ox-LDL led to elevated lipid accumulation in the ECs.

**Impaired autophagy in HUVECs due to ox-LDL.** To investigate the effect of ox-LDL on autophagy in HUVECs, the ratio of LC3-II to LC3-I and the p62 protein expression levels, which serve as well-known biomarkers of autophagy (26), were detected using western blot analysis. As shown in Fig. 3, the results demonstrated that compared with LDL exposure, the ratio of LC3II/LC3I was significantly upregulated in HUVECs exposed to ox-LDL for 6 h, along with significantly increased protein expression levels of LAMP1 and decreased protein levels of p62, suggesting that autophagy events may be triggered in the early period of oxidative stress exposure in ECs. However, compared with the control group, decreased protein levels of LAMP1 were observed in HUVECs exposed to ox-LDL for 24 h, which was accompanied by increased p62 expression. Furthermore, there was a significant decrease in both the ratio of LC3II/LC3I and the protein expression levels of LAMP1, along with a significant increase in p62 expression, in HUVECs treated with ox-LDL for 48 h, indicating that autophagy may be inhibited with prolonged exposure of ECs to ox-LDL.

**Suppression of lipophagy in HUVECs treated with ox-LDL.** To clarify the association between ox-LDL-induced autophagy deficiency and lipid accumulation in ECs, the changes in the number of autolipophagosomes (ALPs) were detected using TEM. The results in Fig. 4 revealed that ALP formation in the ox-LDL treatment group was higher compared with that in the LDL treatment group following 6 h of treatment. However, this ultra-structural conformation was decreased with exposure to

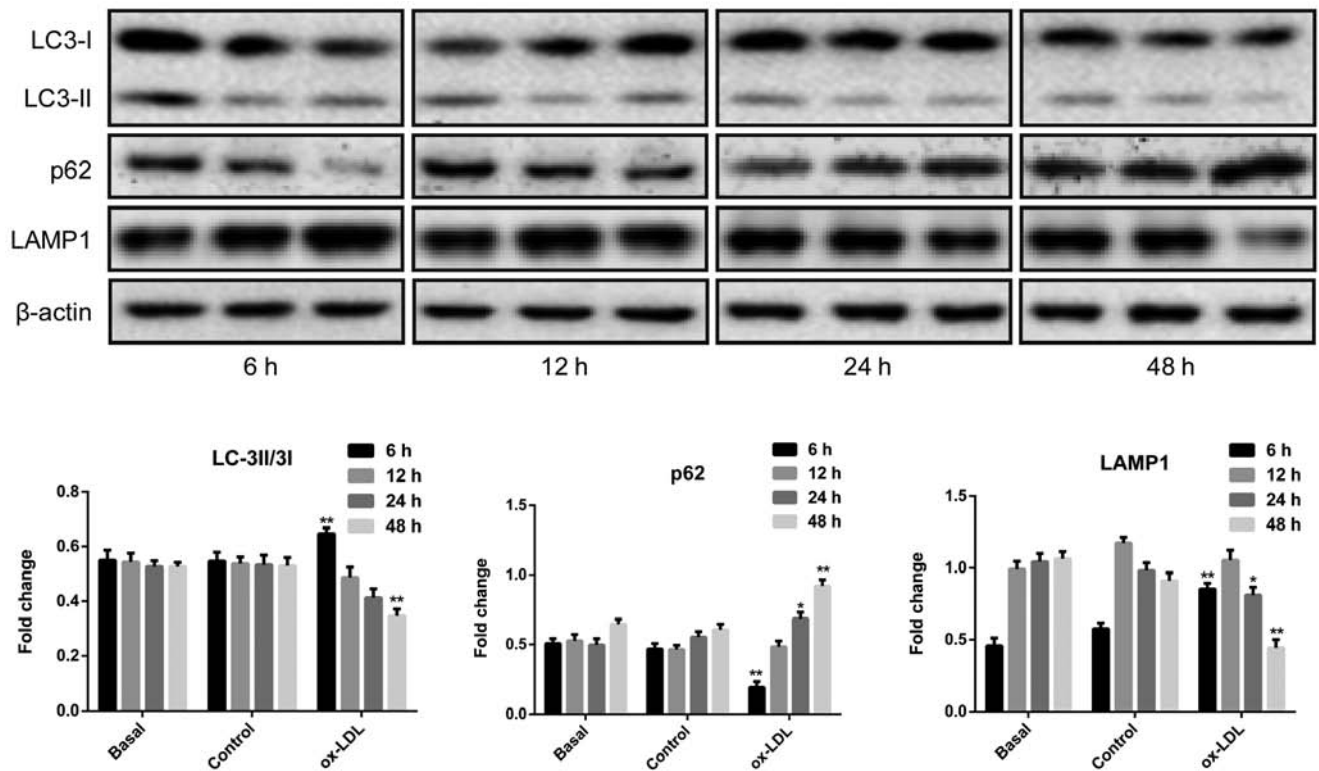


Figure 3. Effect of ox-LDL on the expression of LC3-II/LC3-I, p62 and LAMP1 in HUVECs. HUVECs were treated with either PBS (basal group), 50  $\mu$ g/ml LDL (control group) or 100  $\mu$ g/ml ox-LDL for 6, 12, 24 or 48 h. Expression levels of autophagy-associated proteins (LC3II/I and p62) and LAMP1 were analysed using western blot analysis. The ratio of LC3-II/LC3-I, p62 protein expression and LAMP1 protein expression were quantified using the Fiji software. \* $P < 0.05$ ; \*\* $P < 0.01$  vs. control group ( $n = 3$ ). HUVEC, endothelial cell; ox-LDL, oxidized low-density lipoprotein; LAMP1, lysosomal-associated membrane protein 1.

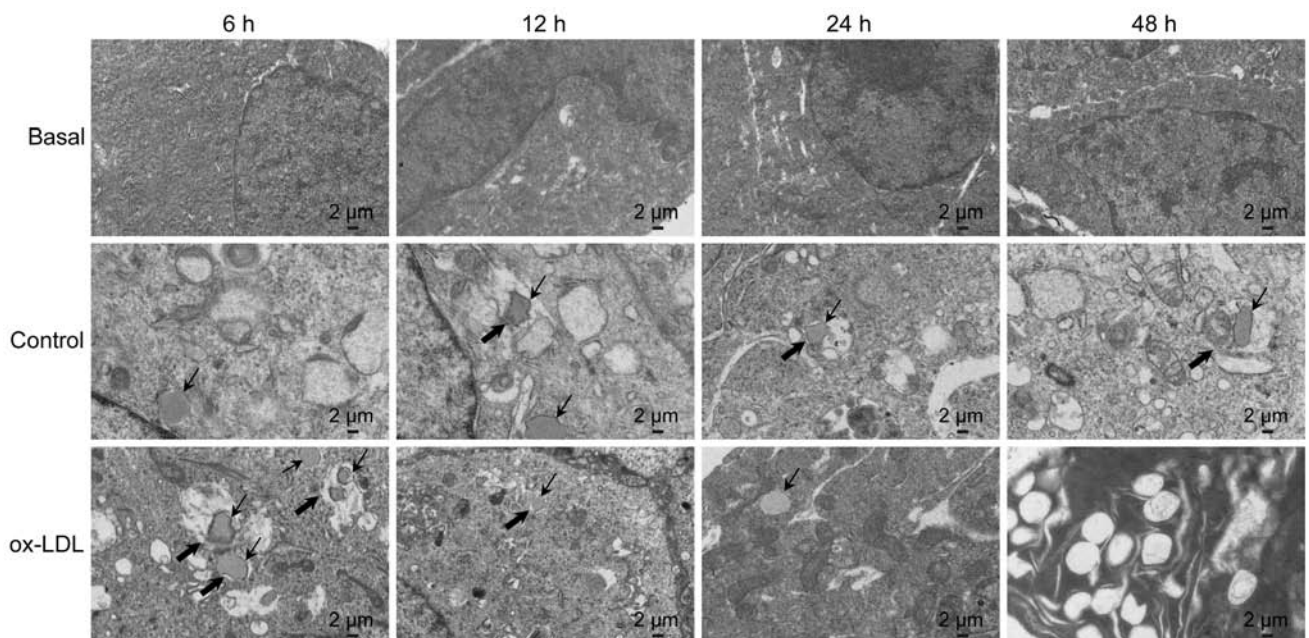


Figure 4. Reduction of lipophagy in HUVECs when exposed to ox-LDL. HUVECs were untreated (basal group) or treated with either 50  $\mu$ g/ml LDL (control group) or 100  $\mu$ g/ml ox-LDL for 6, 12, 24 or 48 h. Scale bar, 2  $\mu$ m. Thick arrows indicate autolipophagosome formation, and thin arrows indicate lipid droplets ( $n = 3$  per group). HUVEC, endothelial cell; ox-LDL, oxidized low-density lipoprotein.

ox-LDL for 24 and 48 h compared with that in LDL-treated HUVECs. In addition, following exposure to ox-LDL for 48 h, the signs of damage was observed in ECs by the presence of

vacuole-like structures. The present results suggested that lipophagy may be impaired after prolonged exposure to oxidative stress.

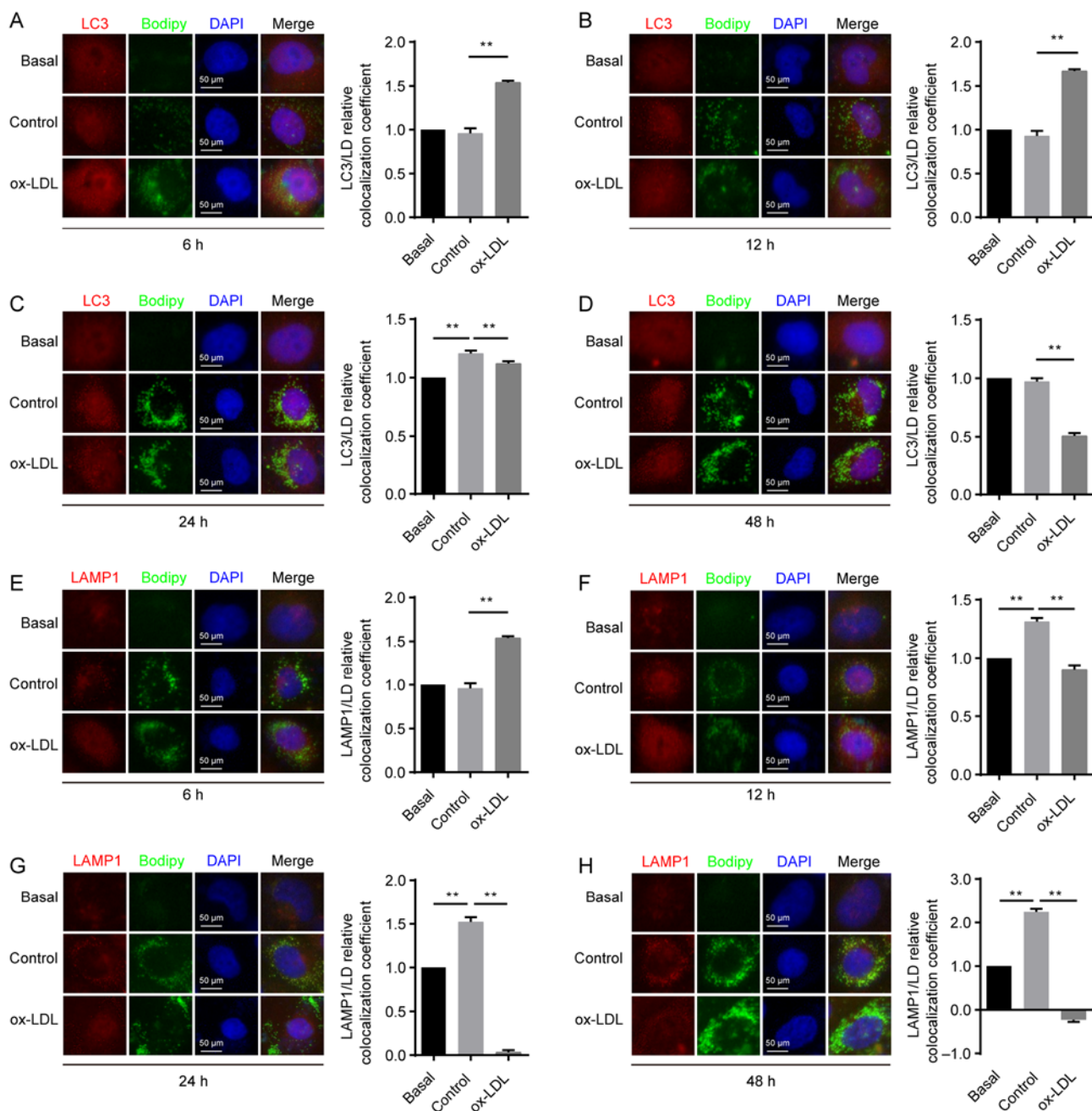


Figure 5. LD delivery to lysosomes for degradation is impaired in HUVECs exposed to ox-LDL. HUVECs were untreated (basal) or treated with either 50  $\mu\text{g/ml}$  LDL (control group) or 100  $\mu\text{g/ml}$  ox-LDL for 6, 12, 24 or 48 h. Co-localization of LC3 (red) and LDs (green) in HUVECs following exposure to ox-LDL for (A) 6, (B) 12, (C) 24 and (D) 48 h. Co-localization of LAMP1 (red) and LDs (green) in HUVECs following exposure to ox-LDL for (E) 6, (F) 12, (G) 24 and (H) 48 h. Magnification,  $\times 400$ . \*\* $P < 0.01$  ( $n = 3$ ). HUVEC, endothelial cell; ox-LDL, oxidized low-density lipoprotein; LD, lipid droplet; LAMP1, lysosomal-associated membrane protein 1.

*Impaired lipophagy may be associated with lipid accumulation in ECs.* To further elucidate the localization of LDs in HUVECs treated with ox-LDL, the co-localization of LDs with LC3 or LAMP1 was measured using confocal microscopy, as previously described (27). Compared with the basal group, the co-localization of LDs with LC3 was significantly increased in HUVECs treated with LDL for 24 h (Fig. 5C). In addition, this co-localization was increased with exposure to ox-LDL for 6 and 12 h compared with that in the control group, (Fig. 5A and B); however, decreased co-localization of LDs with LC3 was observed with exposure to ox-LDL for 24 and 48 h (Fig. 5C and D), indicating that oxidative stress may

reduce lipophagy in HUVECs when treated with ox-LDL for a longer time.

As the exposure time of HUVECs to LDL was increased, the co-localization of LDs with LAMP1 was significantly increased at both 12 and 24 h compared with that in the basal group (Fig. 5E-H), which demonstrated that treatment with natural LDL may accelerate the process of lipid degradation in the lysosomes. Compared with the control group, the co-localization of LDs with LAMP1 was significantly increased in ECs treated with ox-LDL for 6 h (Fig. 5E); however, this co-localization was significantly decreased when increasing the exposure time from 12 to 48 h (Fig. 5F, G and H). The

present results demonstrated that ox-LDL induced by oxidative stress may hinder lipid degradation in the ECs lysosomes.

Overall, the increased lipophagy resulting from exposure to ox-LDL for 6 to 12 h may be associated with enhanced degradation of ox-LDL via autophagy-lysosomal pathways, and the decreased lipophagy observed with exposure to ox-LDL for 24 and 48 h may be associated with attenuated degradation of ox-LDL via autophagy-lysosomal pathways. This phenomenon can be explained by the fact that the lysosomal pathway is associated with decreased ox-LDL degradation, while lipids are degraded in autophagy-lysosomal pathways (28).

## Discussion

AS is a major cause of cardiovascular diseases and typically leads to stroke, myocardial infarction and coronary heart disease (2). Damaged ECs lose the barrier function of the arterial endothelium, accelerating the development of AS (29). Furthermore, ECs are highly sensitive to ox-LDL-induced oxidative damage (30), which is associated with the risk of coronary heart disease and myocardial infarction (31). In addition to being an oxidative stress factor, ox-LDL particles are recognized and captured by scavenger receptors on ECs, rather than by LDL receptors, via a reverse cholesterol transport mechanism (32). It has been previously demonstrated that ox-LDL promotes apoptosis, which disrupts the integrity of the arterial endothelium (33).

The present study demonstrated that exposure to a high concentration of ox-LDL (100  $\mu\text{g/ml}$ ) decreased the viability of HUVECs and increased lipid deposition, whereas natural LDL had no effect on the activity of HUVECs. The present results suggested that oxidative stress may damage ECs, further leading to lipid accumulation. It has been established that 7-keto cholesterol (7KC), one of the components of ox-LDL which has a similar effect on ECs to total ox-LDL (34), is also found in human atherosclerotic plaques and serves an active role in plaque development via inducing vascular EC apoptosis (35). Therefore, it is important to develop strategies to reduce the lipid accumulation induced by ox-LDL.

Both autophagy and apoptosis are associated with maintaining normal cell functions (36). Autophagy inhibits apoptosis to ensure cell survival and it is also an alternative mechanism for cell death in the absence of cell apoptosis (37). In addition, autophagy can protect various vascular cells, such as macrophages and vascular smooth muscle cells, against oxidative stress and inflammation, slowing down atherosclerotic processes (38). It has been demonstrated that autophagy can be induced by 7KC to inhibit the death of smooth muscle cells and to avoid thinning of the fibrous cap (39). Furthermore, it has been revealed that ox-LDL inhibits the autophagy of ECs, serving an essential role in the development of atherosclerotic lesions (12). However, the precise mechanism by which ox-LDL inhibits autophagy in ECs has not yet been elucidated. In the present study, the potential role of autophagy in regulating ox-LDL-induced ECs was investigated. It was revealed that when treating HUVECs with ox-LDL for 24 and 48 h, the ratio of LC3-II/I protein expression levels were significantly decreased, but the protein expression

levels of p62 were significantly increased, indicating that autophagy was significantly impaired. After autophagy is induced, autophagosomes are fused with lysosomes to form autolysosomes, in which the cargo of the autophagosomes is ultimately degraded (40). LAMP1 is an essential factor that mediates the fusion between autophagosomes and lysosomes (16). Treatment with ox-LDL for 24 and 48 h revealed that LAMP1 expression was reduced in HUVECs, which is consistent with previous research (25). Conversely, previous studies demonstrate that autophagy induced by rapamycin can decrease ox-LDL accumulation in ECs (41-43). Overall, the present data indicate that autophagy may be inhibited by ox-LDL in ECs.

Previous studies have revealed that autophagy and lipid accumulation serve important roles in the development of atherosclerotic vascular diseases (44,45). Of note, lipid accumulation has been revealed to accelerate AS development (45); however, the exact mechanism of this process remains unclear. In the present study, the underlying role of autophagy in regulating ox-LDL-induced EC lipid accumulation was explored, and the intermolecular interactions were investigated. Increased lipid staining was observed around the nucleus of HUVECs treated with ox-LDL for 24 and 48 h, suggesting that there may be an association between autophagy and lipid accumulation in ECs exposed to ox-LDL.

Lipophagy is a special form of autophagy (46), which may be involved in transporting LDs from cells to extracellular HDL. To investigate whether lipophagy was impaired under ox-LDL prolonged exposure, numerous ultrastructural changes in ECs were observed using TEM. Compared with control cells, fewer ALPs were observed in cells following treatment with ox-LDL for 24 and 48 h, after which they also exhibited features of cell damage, such as an excess in the number of vacuoles. The present results suggested that lipophagy may be attenuated by ox-LDL, which subsequently promoted lipid accumulation in ECs.

To confirm the impaired lipophagy, the co-localization of LC3, LDs and LAMP1 were assessed using immunofluorescence staining in HUVECs. Compared with the basal group, the co-localization of LDs with LC3 was not significantly different in HUVECs treated with LDL for 6, 12, and 48 h, which suggested that lipophagy occurs under normal LDL exposure. Whereas following treatment with ox-LDL for 24 and 48 h, the co-localization of LC3 and LDs was significantly decreased, as well as the co-localization of LAMP1 and LDs. The present result suggested that endothelial lipophagy may partially maintain vascular lipid homeostasis on autophagosomal-mediated delivery of lipids to the lysosome for degradation (19). Furthermore, lipophagy accelerates lipid hydrolysis, which indirectly promotes the release of free cholesterol to extracellular Apolipoprotein (Apo) AI receptors (47,48).

In conclusion, compared with the control group, lipophagy was impaired in HUVECs treated with ox-LDL, resulting in lipid accumulation. It was hypothesized that lipophagy may be attenuated by ox-LDL, resulting in excess cholesterol deposition in ECs, which may be attributed to inhibiting cholesterol efflux to Apo AI receptors. Overall, the regulation of lipophagy may be an attractive therapeutic strategy to limit atherosclerotic disease in the future.

## Acknowledgements

The authors would like to thank Dr Zhi-xin Huang (Guangdong Second Provincial General Hospital, Guangzhou, China) for assisting with statistical analysis; Dr Hong An (University of Missouri, Columbia, MO, USA) for helping to modify the manuscript; Dr Tan Tan and Dr Xiao-bo Hu (University of South China, Hengyang, China) for technical support.

## Funding

The present study was supported by the National Nature Science Fund of China (grant nos. 81600291 and 31871169), the Nature Science Fund of Hunan province (grant nos. 2018JJ2346 and 2018JJ2348) and the Hunan Provincial Innovation Foundation for Postgraduate (grant no. CX2018B62).

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

SYL, MZ and CPZ conceptualized and designed the experiments. XXD, TT and CPZ performed the experiments. BJL, CYW and SSJ analyzed and interpreted the data. JQS and YLY acquired the data. CPZ and XXD wrote the manuscript. YT made substantial contributions to conception of the study and critically revised the manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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