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The protease inhibitor E64d improves ox-LDL-induced endothelial dysfunction in human aortic endothelial cells

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Abstract

Oxidized low-density lipoprotein (Ox-LDL)-induced endothelial dysfunction in human vascular endothelial cells contributes to the development of atherosclerosis. E64d, a cysteine protease inhibitor, blocks the elastolytic activity of cathepsin essential for vascular matrix remodeling and reduces neurovascular endothelial apoptosis. The objective of this study was to investigate the effects and the underlying mechanisms of E64d on ox-LDL-induced endothelial dysfunction in human aortic endothelial cells (HAECs). HAECs were treated with various concentrations of ox-LDL (0-200 mg/L) for 24h with or without E64d. The results showed that E64d attenuated ox-LDL-induced increase in soluble intercellular adhesion molecule-1 (sICAM-1) concentration and reduction in endothelial nitric oxide synthase (eNOS) expression, prevented ox-LDL-induced reduction in cell viability and migration ability of HAECs. E64d decreased the protein expression of cathepsin B (CTSB), Beclin 1 and microtubule-associated protein light chain 3 (LC3)-II, but not p62. LC3 puncta and autophagosome formation were also reduced by E64d in HAECs. Moreover, E64d decreased the production of MDA and increased the activity of SOD. In conclusion, E64d ameliorated ox-LDL-induced endothelial dysfunction in HAECs.

Keywords: E64d, endothelial dysfunction, ox-LDL, autophagy, HAECs, atherosclerosis, oxidative stress

Draft

Introduction

Atherosclerosis, the leading cause of death and morbidity worldwide, is a disease characterized by imbalanced lipid metabolism and chronic inflammation (Weber and Noels 2011). Endothelial cells cover the inner surface of all blood vessels, forming a physical barrier to protect against atherosclerosis (Förstermann and Münzel 2006). In physiological conditions, endothelial cells properly regulate vascular tone and inflammation, prevent thrombosis, and maintain vascular integrity (Aird 2007). When endothelial cells are exposed to risk factors such as high blood pressure, elevated glucose, or elevated lipids, endothelial dysfunction occurs, with reduced expression of eNOS (Förstermann and Münzel 2006), increased production of adhesion molecules, including ICAM-1 (Chapman and Sposito 2008), and disrupted migratory ability (Pirillo et al. 2013). As a key component in the hyperlipidemic state, ox-LDL induces endothelial dysfunction by increasing oxidative stress in endothelial cells (Zhang et al. 2016; Galle et al. 2006; Davignon et al. 2004).

E64d is a potent small-molecule inhibitor of papain-like cysteine proteases and calcium-activated neutral proteases, which include cathepsin B (CTSB) (Tamai et al. 1986). Hence, E64d has been implicated in the regulation of autophagy, a lysosome-mediated intracellular degradation pathway

(Hwang et al. 2015; Le Fourn et al. 2009; Ni et al. 2011). Autophagy is a highly conserved process in eukaryotes. In the initial step of this process, cytoplasmic components are enveloped and sequestered by double-membrane vesicle called autophagosome, and then the autophagosome fuses to lysosomes for degradation (Komatsu et al. 2007; Watson et al. 2012). A large body of evidences indicate that E64d is efficacious in different preclinical pathological models, such as atherosclerosis (Sukhova et al. 1998), Alzheimer's disease (Hook et al. 2011), brain ischemia (Tsubokawa et al. 2006), rheumatoid arthritis (Yoshifuji et al. 2005) and multiple myeloma (Tibullo et al. 2016). E64d blocks the extracellular degradation of elastin, which is essential for vessel wall remodeling during atherosclerosis (Sukhova et al. 1998), and attenuates neurovascular endothelial apoptosis in rats with cerebral ischemia (Tsubokawa et al. 2006). These studies suggest that E64d might protect endothelial cells and prevent atherosclerosis. The potential effects of E64d on ox-LDL-induced endothelial dysfunction are, however, not yet known. Therefore, the present study was performed to investigate the effects and the underling mechanisms of E64d on ox-LDL-induced endothelial dysfunction in HAECs.

Materials and methods

Reagents and antibodies

Human ox-LDL was purchased from Peking Union-Biology (Beijing, China). E64d was purchased from Sigma-Aldrich (St. Louis, MO, USA). Endothelial cell medium (ECM), fetal bovine serum (FBS), endothelial cell growth supplement (ECGS), and penicillin/streptomycin solution (P/S) were purchased from ScienCell Research Laboratories (San Diego, CA, USA). MDA and SOD assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Human sICAM-1 Quantikine ELISA Kit was purchased from R&D systems (Minneapolis, MN, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The anti-LC3 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-SQSTM1/p62 antibody was purchased from Abcam (Massachusetts, USA). Anti-eNOS antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Beclin 1 antibody was purchased from Novus Biologicals (Littleton, CO, USA). Anti-Cathepsin B antibody was purchased from R&D Systems. Anti- β -actin antibody was purchased from ZSGB-BIO (Beijing, China). Goat anti-rabbit IgG conjugated with Chromeo-546 secondary antibody was purchased from Abcam (Cambridge, UK).

Cell culture

Primary cultures of HAECs were purchased from ScienCell Research Laboratories. Cells were cultured in ECM supplemented with 5% FBS, 1% ECGS, and 1% P/S at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were placed into T75 plastic flasks and 6-well plates for experiments. Complete culture medium was replaced every 3 days. HAECs were used between passages 3 to 5.

Cell viability by CCK-8 assay

The CCK-8 cell proliferation assay was used to measure cell viability according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates at an appropriate density of cells/well and incubated at 37 °C for 12h. Then cells were treated with ox-LDL (0, 50, 100, 150, 200 mg/L) with or without 1 µM E64d for 24h. Subsequently, CCK-8 reagent (10 µL/well) was added and the plate was incubated at 37 °C for 2h. The absorbance was determined with a microplate reader (Tecan Austria GmbH, Austria) at a wavelength of 450 nm.

Enzyme-linked immunosorbent assay (ELISA)

To detect the concentration of sICAM-1, MDA and SOD in the culture medium, the medium was collected from each culture flasks at the end of each period. Following a gentle wash with sterile

phosphate-buffered saline (PBS), cells were digested with trypsin and then counted by CASY[®] Cell Counter and Analyser System, Model TTC (Schärfe Systems GmbH, Reutlingen, Germany). The sample was measured by ELISA-kits according to the manufacturer's instructions. Absorbance was detected with a microplate reader at 450 nm. All results were adjusted cell counts.

Wound healing assay

A wound healing assay was performed to test cell migration. Cells were seeded in 6-well plates and grown to form a confluent monolayer. A wound was made using a 10-20 μ L plastic pipette tip and marked on the top of the plate. Cellular debris was removed by gentle wash with culture medium, and then cells were treated with control, or 150 mg/L ox-LDL and 1 μ M E64d followed by 150 mg/L ox-LDL for 24h in a humidified incubator at 37 °C. Images were taken under the light microscope based on markers on the top of the plate, which allowed for a comparison of each of the image at different time points. The gap size was analyzed using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Immunofluorescence

Cells grown to about 70% confluence on coverslips were treated with control, or 150 mg/L ox-LDL

and 1 μM E64d followed by 150 mg/L ox-LDL for 24h in a humidified incubator at 37 °C. Then cells were washed gently with PBS and fixed in 4% paraformaldehyde for 30 min. After being washed with PBS, cells were blocked with PBS containing 3% bovine serum albumin (BSA) for 15 min. Next, cells were washed with PBS and incubated overnight at 4 °C with primary antibody (anti-LC3, 1:200) diluted in PBS-3% BSA. After staining with a fluorescent secondary antibody, cells were incubated in the dark for 30 min at room temperature, and washed with PBS. Finally, cells were visualized under a confocal fluorescence microscope (TCS-Sp5, Leica Microsystems, Germany).

Transmission electron microscopy (TEM)

For ultrastructural analysis, cells were cultured in T75 plastic flasks and treated with control, or 150 mg/L ox-LDL and 1 μM E64d followed by 150 mg/L ox-LDL for 24h. Cells were harvested and centrifuged at 1000 rpm for 10 min, chemically fixed overnight at 4°C in 4 % glutaraldehyde and processed for TEM.

Western blot analysis

Western blot analysis was used to detect LC3, p62, Beclin 1, eNOS and CTSB protein expression. Protein concentrations of the cells were determined using BCA protein assay kit (Pierce, Rockford, IL,

USA). Equal amounts of protein (20µg) were separated by 8% or 15% SDS-PAGE electrophoresis and then transferred to a polyvinylidene difluoride membrane by electroblotting. The membranes were blocked with 5% nonfat skim milk for 1h and then incubated with primary antibodies (LC3, 1:1000; p62, 1:1000; Beclin 1, 1:1500; eNOS, 1:200; CTSB, 1:2000) overnight at 4°C. After incubation with secondary antibody (1:1500) at room temperature for 1h, the membranes were washed. Antigen-antibody complex was detected using the Enhanced Chemiluminescence kit (Pierce, Rockford, IL, USA). The densitometric analysis was performed using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are expressed as mean $\pm$ SD. Comparisons between groups were performed by one-way ANOVA analysis. Values of $p < 0.05$ were considered statistically significant.

Results

Ox-LDL induced endothelial dysfunction and reduced autophagy flux in HAECs

In the present study, we investigated the oxidative stress response of HAECs after treatment with ox-LDL (0, 50, 100, 150, 200 mg/L) by measuring the concentration of oxidative stress markers,

MDA and SOD. Ox-LDL increased the production of MDA, and decreased the activity of SOD in a dose-dependent manner (Fig.1A and 1B). Moreover, we measured the effect of ox-LDL on the secretion of inflammatory cytokines. sICAM-1 secretion was increased after ox-LDL exposure in a dose-dependent manner (Fig.1C). The protein level of eNOS was decreased after ox-LDL treatment (Fig.1D). We examined the viability of the dose-effect of ox-LDL on HAECs. When the ox-LDL concentration reached 150 mg/L, the viability of cells was significantly decreased to 50% compared with control (Fig.1E). Therefore, 150 mg/L ox-LDL was selected as the optimal concentration for subsequent experiments. The autophagy marker LC3 and autophagic substrate p62 protein expressions were dose-dependently upregulated in response to ox-LDL (Fig.1F and 1G).

E64d ameliorated ox-LDL-induced endothelial dysfunction in HAECs

E64d greatly attenuated ox-LDL-induced increase in sICAM-1 concentration and reduction in eNOS expression (Fig. 2A and 2B). In the wound healing assay, E64d significantly accelerated cell migration into the wounded area compared with ox-LDL treatment alone (Fig. 2C). The effect of ox-LDL in reducing cell viability was prevented by E64d (Fig. 2D).

E64d reduced ox-LDL-induced autophagy and oxidative stress in HAECs

The protein expression of CTSB was increased in cells exposed to ox-LDL (150 mg/L), whereas E64d (1 μ M) inhibited the ox-LDL-induced increase in CTSB expression (Fig. 3A). Moreover, E64d reduced the protein level of autophagy marker LC3 and increased p62 compared with ox-LDL treatment alone (Fig. 3B and 3C). In addition, E64d decreased the protein level of Beclin 1 in cells incubated with ox-LDL (Fig. 3D). In order to obtain further insight regarding the effect of E64d on ox-LDL-induced autophagy, immunofluorescence and transmission electron microscopy were performed. After treatment with ox-LDL, an increase of LC3 puncta representing autophagic vacuoles was formed in the cytoplasm. When E64d was added to culture medium, the ox-LDL-induced increase in LC3 puncta was significantly reduced (Fig. 3E). Electron microscopy images showed untreated cells with normal cytoplasmic and nuclear morphology (Fig. 3F-a). Numerous double-membranous vacuoles engulfing parts of the cytoplasm were observed in the ox-LDL-treated cells (Fig. 3F-b). However, E64d attenuated the ox-LDL-induced increase in double-membranous vacuoles (Fig. 3F-c). Furthermore, E64d decreased the production of MDA and increased the activity of SOD in cells compared with ox-LDL treatment alone (Fig. 3G and 3H).

Discussion

In this study, we provide a new insight into the effects of E64d on ox-LDL-induced endothelial dysfunction in HAECs. The results showed that E64d treatment reduced the production of the adhesion molecule sICAM-1 and enhanced eNOS protein expression, migration ability and cell viability injured by ox-LDL in HAECs.

Ox-LDL-induced endothelial dysfunction plays a key role in the initiation and development of atherosclerosis (Zhang et al. 2016; Tsai et al. 2011). Interaction of ox-LDL with endothelial cells via lectin-like oxidized LDL receptor 1 (LOX-1), the major endothelial receptor for ox-LDL, generates superoxide anion. In a second step, excess superoxide anion accelerates rapid oxidative inactivation of nitric oxide and induces eNOS dysfunction. eNOS dysfunction produces superoxide anion instead of NO, which partially aggravate oxidative stress (Masaki 2003; Li et al. 2013). Abundant evidences indicate that oxidative stress is a major inducer of autophagy, which involved in various biological and pathological processes (Filomeni et al. 2010; Chen et al. 2009; Kromemr et al. 2010; Komatsu et al. 2007).

Inhibition of endothelial dysfunction has become a critical target for preventing or slowing the progression of atherosclerosis. Ox-LDL induces endothelial dysfunction by increasing oxidative stress,

which appears to be a common denominator in atherosclerosis (Lu et al. 2011; Félétou and Vanhoutte 2006). Our present study showed that ox-LDL dose-dependently caused the production of MDA and the reduction of SOD, which suggests that the oxidative stress was activated in HAECs. Subsequently, we found that the protein levels of LC3-II and p62 were increased under oxidative stress, indicating autophagy was stimulated while autophagy flux was impaired. Meanwhile, we found that ox-LDL decreased cell viability and eNOS protein expression and increased the sICAM-1 production.

Autophagy - the internal digestion of parts of a cell - is an evolutionarily conserved pathway for recycling damaged or unwanted cellular components (Rubinsztein 2015). At a basal rate, autophagy acts as a major housekeeping mechanism, crucially involved in the maintenance of normal cellular homeostasis (Zhang et al. 2016). The role of autophagy in atherosclerosis seems to be complex. It has been demonstrated that defection of the physiological level of autophagy results in markedly increased atherogenic inflammasome activation and atherosclerotic plaque formation (Razani et al. 2012; Liao et al. 2012). Such effects are also bolstered by observations from another study, in which upregulation of autophagy promoted ox-LDL degradation in endothelial cells (Zhang et al. 2016). As per the adage, “excess of anything is bad”. Apart from its protective effects, autophagy may also

accelerate atherosclerosis progression and clinical complications when it is excessively activated.

Excessively-activated autophagy may cause autophagic smooth muscle cell death, which in turn results in plaque destabilization because of the decreased synthesis of collagen and thinning of the fibrous cap (Levine and Yuan 2005). How can autophagy keep the balance between detrimental and protective effects to continuously protect cells? One possibility is that the level of autophagy is properly, which cannot be separated from subtle regulation involved in the intricate signaling network.

p62 accumulates when autophagy is inhibited, and decreases when autophagy is induced, therefore, it is usually considered as a marker of autophagy flux (Hwang et al. 2015). In this study, the protein level of autophagic substrate p62 was apparently increased after treatment with E64d, suggesting that autophagy flux was decreased. It is now generally accepted that reduced autophagy flux is deleterious (Hwang et al. 2015; Xu et al. 2013). A puzzling aspect of this study was increased protein expression of p62 accompanied by reduced LC3-II expression and ameliorated endothelial dysfunction after treatment with E64d. The potential explanation for this conundrum is that p62, in addition to its role as a signaling hub whose levels can be regulated by autophagy, may also be a central element in a quality-control mechanism for the disposal of toxic aggregates as previously suggested (Moscat and

Diaz-Meco 2009). It is believed that p62 accumulation triggers the synthesis of a number of detoxifying enzymes to prevent oxidative stress (Moscat et al. 2016). The findings of Duran and coworkers provide evidence that the reduced NF- κ B activation observed in the p62-deficient cells leads to lower levels of ROS scavengers, which results in enhanced ROS levels and more cell injury (Duran et al. 2008). More data demonstrate that p62, due to its location on lysosomes and its ability to bind Raptor and the Rag proteins, regulates mTORC1 activity (Duran et al. 2011). Since mTORC1 stimulates several anabolic pathways that promote cell growth and proliferation, these findings reveal that p62 not only controls cell survival of normal cells, but also contributes to cell growth (Moscat et al. 2016). These unexpected findings imply that p62, which is degraded by autophagy, also regulates autophagy (Moscat and Diaz-Meco 2011). These might explain E64d reduced LC3-II protein expression and oxidative stress and endothelial dysfunction which were induced by ox-LDL, while protein level of p62 was increased in HAECs.

The cysteine protease inhibitor E64d is a membrane-permeable inhibitor of cathepsins B, L, H, K, S, F, O, V, W, X, calpains 1 and 2, and papain (Hashida et al. 1980). Lysosomal cysteine proteases, in particular the highly-expressed CTSB, have important functions in autophagy (Stoka et al. 2016). In

view of its inhibitory effect on lysosomal cysteine proteases, E64d was treated as an autophagy inhibitor in several studies (Le Fourn et al. 2009; Ni et al. 2011; Zhang et al. 2013). E64d, as an autophagy inhibitor, reduces seizure-induced brain damage by modulating lysosomal cathepsins L and D in rats (Ni et al. 2016), blocks large number of cell death under nutrition deprivation (Xu et al. 2013), and attenuates ARV-mediated apoptosis (Duan et al. 2015). However, whether the effect of E64d on the level of autophagy by modulating CTSB could protect against ox-LDL-induced endothelial dysfunction is still unknown. LC3 is a modifier protein conjugated with phosphatidylethanolamine (PE). LC3-I (the non-lipidated form of LC3, which does not participate in autophagy) conversion to LC3-II (PE-conjugated LC3) is essential for the formation of autophagosomes and it is used as a marker to monitor autophagy (Kimmey et al. 2015). Beclin 1 is a core subunit of the autophagy initiation complex involved in autophagosome formation (He and Levine 2010). In this study, we found that E64d decreased CTSB and LC3-II and Beclin 1 protein levels with improving cell viability, migration ability, eNOS protein expression and sICAM-1 production, indicating that restoration of endothelial cell function by E64d may be related to the degree to which autophagy is reduced. In the study of E64d on autophagy, Dong and coworkers have

found that E64d attenuated induction of LC3-II and Beclin 1 (Dong et al. 2012). Consistent with the western blot data in the present study, E64d lowered the number of LC3 puncta in ox-LDL-induced endothelial cells. However, there is a study of E64d showed that both LC3-II and p62 protein expressions were increased after treatment with E64d as an autophagy inhibitor (Ni et al. 2011). Considering the different cell types, treatment factors, experimental models, and environmental conditions appear to dramatically influence the effects of E64d.

These findings enrich our understanding of the beneficial effect of E64d on endothelial dysfunction during atherosclerosis. Our data also indicate that manipulation of an appropriate level of autophagy might be a promising preventive and therapeutic target for the treatment of atherosclerosis.

In consideration of the level of autophagy as well as oxidative stress was decreased when HAECs were treated with E64d. We could not overlook the possibility that E64d interferes with release of ox-LDL components after particle uptake by LOX-1 on HAECs, thereby reducing oxidative stress, and induction of autophagy finally. Unfortunately, we could not determine the explicit mechanism in the present study, which is a major limitation of the current study, therefore, further studies are needed to define the underlying mechanism of the effects.

In conclusion, the results of the present study indicate that E64d ameliorates, at least in part, ox-LDL-induced endothelial dysfunction related with sICAM-1, eNOS, MDA, SOD, cell viability and migration ability in HAECs.

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Conflict of interest

No conflicts of interest, financial or otherwise, are declared by any of the authors.

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Figure captions

Figure 1. Effect of various concentrations of ox-LDL on endothelial cell function and autophagy flux in HAECs. Treatment of HAECs with ox-LDL at various concentrations for 24h. (A, B and C) MDA, SOD and sICAM-1 concentrations in culture medium were measured using ELISA. (D) Protein level of eNOS was determined by Western blot and quantification analysis. β -actin served as loading controls. (E) Cell viability was assessed using CCK-8 assay. Cell viability was given as a percentage of control. (F and G) Protein expression of LC3 and p62 were determined by Western blot and quantification analysis. β -actin served as loading controls. Data were expressed as mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. control.

Figure 2. Effect of E64d on ox-LDL-induced endothelial dysfunction in HAECs. HAECs were treated

with control, or 150 mg/L ox-LDL and 1 μ M E64d followed by 150 mg/L ox-LDL for 24h. (A)

sICAM-1 concentrations in culture medium were measured by using ELISA. (B) Level of eNOS was

determined by Western blot and quantification analysis. β -actin served as loading controls. (C)

Migration of cells was detected by wound healing assay. Scale bar: 100 μ m. (D) Cell viability was

assessed using CCK-8 assay. Cell viability was given as a percentage of control. Data were expressed

as mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. ox-LDL treatment

alone.

Figure 3. Effect of E64d on ox-LDL-induced autophagy and oxidative stress in HAECs. HAECs were

treated with control, or 150 mg/L ox-LDL and 1 μ M E64d followed by 150 mg/L ox-LDL for 24h. (A,

B, C and D) Protein expression of CTSB, LC3, p62 and Beclin 1 was determined by Western blot and

quantification analysis. β -actin served as loading controls. (E) Fluorescence signals were visualized by

confocal fluorescence microscopy. Cultures were stained with LC3 (red) and the nuclear marker DAPI

(blue). Scale bar: 20 μ m. The average number of LC3 puncta in each cell was determined from at least

40 cells in each group. (F) Electron microscopy images showed ultrastructures of HAECs from each

group. (F-a) Untreated cell, Scale bar: 1 μ m; (F-b) ox-LDL treatment: 150 mg/L ox-LDL for 24h, Scale

bar: 1 μm ; (F-c) E64d+ox-LDL treatment: 1 μM E64d and 150 mg/L ox-LDL for 24h, Scale bar: 1 μm .

Black arrow points to double-membraned autophagosomes in the cytoplasm. (G and H) MDA and

SOD concentrations in culture medium were measured using ELISA. Data were expressed as mean $\pm$

SD of three independent experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. ox-LDL treatment alone.

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