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Ox-Lp(a) transiently induces HUVEC autophagy via an ROS-dependent PAPR-1-LKB1–AMPK–mTOR pathway

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ABSTRACT

Oxidised lipoprotein(a) [oxLp(a)] is considered as a more potent arteriosclerotic factor than native Lp(a). However, the molecular mechanisms underlying this potency remain unclear. Reactive oxygen species (ROS) possibly act as intracellular second messengers that participate in autophagy stimulation. In this study, the effect of oxLp(a) on endothelial cell autophagy was determined. The mechanism and effect of autophagy on endothelial cells were also investigated. Results showed that oxLp(a) could induce autophagy depending on the generation of cellular ROS. Superoxide dismutase, an antioxidant, could inhibit oxLp(a)-induced autophagy in human umbilical vascular endothelial cells. Furthermore, poly(adenosine diphosphate-ribose) polymerase-1 (PARP-1)-liver kinase B1 (LKB1)-adenosine monophosphate-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR) and LKB1–AMPK–mTOR pathways are involved in oxLp(a)-induced autophagy. These pathways are also dependent on ROS. Thus, oxLp(a) induced autophagy via LKB1–AMPK–mTOR and PAPR-1-LKB1–AMPK–mTOR pathways, which are dependent on ROS generation.

1. Introduction

Studies have demonstrated that the increased plasma concentration of lipoprotein(a) [Lp(a)] is associated with atherosclerosis; therefore, Lp(a) is considered as an independent risk factor of atherosclerosis [1,2]. Ox-Lp(a) is more easily deposited in coronary calcified areas than Lp(a), as histochemically detected in patients with myocardial infarction [3,4]. OxLp(a) may be more potent than native Lp(a) in promoting atherosclerosis. However, the mechanism of oxLp(a) in atherosclerosis remains unclear. Previous studies showed that oxidative stress is an important mechanism responsible for the actions of oxidised lipids in various biological systems. For example, increased reactive oxygen species (ROS) generation has been detected in oxidised low-density lipoprotein (LDL)-treated endothelial cells [5]. Lp(a) is an LDL-like lipoprotein comprising a cholesterol ester-containing core and apolipoprotein(a) moiety covalently linked to the surface apolipoprotein B component of LDL. Thus, we speculated that the proatherogenic potential of ox-Lp(a) may contribute to its ability to stimulate ROS formation, as demonstrated in a previous study [6].

Oxidative stress is defined as an imbalanced redox state in which pro-oxidants overwhelm antioxidant capacity, resulting in increased ROS production. ROS has been implicated in the pathogenesis of every stage of vascular lesion formation in atherosclerosis [7]. In general, vascular endothelial cells are among the sources of ROS in the vessel wall, and such cells participate in vessel pathology.

Autophagy, which is implicated in housekeeping, is involved in the maintenance of normal cellular homeostasis. When stimulated by cellular stress, autophagy functions as a self-cannibalisation

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pathway that promotes cell survival in an unfavourable environment [8,9]. Thus far, preclinical evidence has suggested that autophagy is associated with atherosclerosis [10,11]. In vitro studies have also identified several potential autophagy stimuli, such as inflammation, ROS production, accumulation of oxidised lipoproteins and endoplasmic reticulum stress; these stimuli are observed in atherosclerotic plaques [12–14]. Kiffin et al. [15] demonstrated that basal autophagy can protect plaque cells against oxidative stress by degrading damaged intracellular materials, particularly polarised mitochondria. In contrast to basal autophagy, excessively stimulated autophagy in endothelial cells may cause autophagic death. Endothelial cell death may be detrimental for the structure of plaque because endothelial injury or death is a primary mechanism of acute clinical events by promoting lesional thrombosis [16,17]. Thus, autophagy acts as a "double sword" in arteriosclerosis.

Previous studies showed that ROS can stimulate autophagy via adenosine monophosphate (AMP)-activated protein kinase (AMPK) signalling, thereby preventing oxidative injury and dysfunction of the vascular endothelium [18]. AMPK, which is a regulator of metabolic abnormality, is a serine–threonine kinase. AMPK is activated by liver kinase B1 (LKB1), an upper kinase [19]. The LKB1-AMPK pathway is necessary to control cellular autophagy under oxidative stress. Furthermore, the mammalian target of rapamycin (mTOR) is an AMPK downstream kinase that negatively regulates autophagy [20]. Therefore, ROS may induce autophagy via the LKB1–AMPK–mTOR pathway.

Under oxidative stress, poly(ADP-ribose) polymerase-1 (PARP-1) is activated and contributes to necrotic cell death via ATP depletion [21]. Moreover, PARP is involved in the autophagy of mouse embryonic fibroblasts and anti-hydrogen peroxide-induced necrotic cell death [22]. In our previous study, oxLp(a) increased ROS generation [6]. Thus, we hypothesised that PARP may be involved in oxLp(a)-induced autophagy.

This study was conducted to explore the signalling pathway linking oxLp(a) and autophagy. We also aimed to investigate the function of autophagy in ROS-induced cell death. We found that oxLp(a) was involved in autophagy, which is partially mediated by PARP-1-LKB1-AMP-mTOR signalling and directly by LKB1-AMP-mTOR signalling. Furthermore, these signalling events were dependent on ROS. Autophagy may also function as a cell survival mechanism against ROS-mediated cell death. Hence, the novel function of oxLp(a) in regulating the autophagy of human umbilical vein endothelial cells (HUVECs) should be understood to elucidate the role of oxLp(a) in arteriosclerosis.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) containing high glucose, trypsin and foetal bovine serum (FBS) was purchased from Hyclone, a part of Thermo Fisher Scientific (Logan, USA). RevertAid™ first-strand cDNA synthesis kit and Liposome 2000 were purchased from Invitrogen (Carlsbad, USA). The antibodies (rabbit anti-human) against PARP-1, poly(ADP-ribose) (PAR), LC3, beclin-1, Bax and Bcl-2 were purchased from Cell Signalling Technology (Beverly, MA, USA). β-actin anti-body (rabbit anti-human) and horseradish peroxidase (HRP)-conjugated second antibody (goat anti-rabbit) were purchased from CWBIO (Peking, China). p-AMPK (Thr172), p-mTOR, p-p70S6K (Thr389), p-4EBP1 (Thr37/46) and AMPK small interfering RNA (siRNA) were purchased from Santa Cruz (CA, USA). AMPK inhibitor Compound C, PPAR-1 inhibitor 3-amino benzamide (3AB), Chloroquine(CQ), monodansylcadaverine (MDC), Annexin V/IP, Lp(a) and superoxide dismutase (SOD) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). GFP-LC3 plasmid was purchased from Genechem (Shanghai, China).

2.2. Cell culture

HUVECs were purchased from Central South University and cultured in HepG2 cells that were cultured in DMEM-high glucose medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml in an incubator under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were cultured without serum for at least 6 h before the experiments were conducted.

2.3. Preparation and identification of oxLp(a)

Dialysis was conducted for 72 h in a bag filter containing pure phosphate-buffered saline (PBS) at 4 °C to remove ethylenediaminetetraacetic acid (EDTA). Afterwards, human Lp(a) was oxidised using 20 μmol/L Cu_2SO_4 (oxidant) in PBS at 37 °C to initiate oxidative effect. After approximately 6 h, Lp(a) was observed from canary to milkiness. Continuous oxidation was performed for 36 h; oxidation was then terminated by adding 20 μmol/L EDTA in PBS at 4 °C for 24 h and renoveaturing the dialysate every 8 h. Oxidative purity was analysed by agarose gel electrophoresis, and the migration of oxLp(a) was compared with that of Lp(a). Lp(a) oxidation proceeded efficiently if the oxLp(a) lot migrated farther than the native Lp(a).

2.4. MDC staining

Autophagocytic vacuoles were stained with 50 μM MDC by incubating cells in 12-well culture plates at 37 °C for 30 min in the dark. The cells were washed thrice with PBS at 4 °C and then fixed in 4% paraformaldehyde for 20 min. Afterwards, the cells were washed thrice with PBS and analysed using a fluorescence microscope excitation filter (340 nm–380 nm; barrier filter, 430 nm; Nikon, Japan).

2.5. Real-time PCR

Real-time PCR was performed using the Light Cycler Fast Start DNA Master SYBR Green kit (Roche, Vienna, Austria) in a Light Cycler 1.0 system. First-strand cDNA was synthesised from total RNA by using a first-strand cDNA synthesis kit (Roche) according to the manufacturer's instructions. The cDNA was initially diluted tenfold and then equal amounts were added to real-time PCRs in duplicate or triplicate. To ensure the highest possible accuracy, we prepared a master mix containing all of reagents except the primers. The master mix was added directly to the glass capillary with the primers of the gene of interest or a housekeeping gene. Each reaction proceeded for 40 amplification cycles; melting curve analysis was then performed to ensure the specificity of each reaction under the control of the supplied Light Cycler software (version 3.5, Roche). Crossing point and melting curve analyses of each reaction were also performed using the Light Cycler software.

2.6. Western blot analysis

The expression of cell proteins was examined by western blot analysis. Cell proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride microporous membrane. The membrane was subsequently blocked with 5% skimmed milk powder in Tris-buffered saline Tween-20 (TBST; 10 mM Tris–HCl, pH 7.6, 150 mM NaCl and 0.05% Tween-20) for 4 h and incubated with the objective primary antibody [β-actin (1:1500), LC3 (1:1000), beclin-