

## Original Article

# Kaempferol alleviates ox-LDL-induced apoptosis by up-regulation of autophagy via inhibiting PI3K/Akt/mTOR pathway in human endothelial cells<sup>☆,☆☆</sup>



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## ABSTRACT

Oxidized low-density lipoprotein (ox-LDL) has been reported to induce apoptosis of endothelial cells (ECs) and contribute to the progression of atherosclerosis. Kaempferol has been shown to possess antiatherosclerotic effect. The aim of the present study was to evaluate the effect of kaempferol on ox-LDL-induced apoptosis of human umbilical vein endothelial cells (HUVECs) and its possible molecular basis. The results showed that kaempferol alleviated ox-LDL-induced apoptosis. Kaempferol increased the ratio of LC3-II/I and beclin-1 level in ox-LDL-induced HUVECs. Moreover, the expression of p-Akt and p-mTOR was down-regulated after treatment with kaempferol in ox-LDL-treated HUVECs, which is similar to the effect of PI3K inhibitor (LY294002) or mTOR inhibitor [rapamycin (RAP)]. Besides, autophagy induced by kaempferol was enhanced by LY294002 or RAP, while kaempferol-induced autophagy was attenuated with insulin treatment, the activator of PI3K/Akt/mTOR pathway. Furthermore, insulin also abated the effect of kaempferol on cell viability and apoptosis in ox-LDL-induced HUVECs. The results indicated that kaempferol alleviated ox-LDL-induced cell apoptosis by up-regulation of autophagy via inhibiting PI3K/Akt/mTOR pathway in human ECs.

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## 1. Introduction

Atherosclerosis, a kind of chronic vascular disease with high morbidity and mortality worldwide, is a leading cause of heart disease and stroke [1,2]. A variety of studies have focused on the pathophysiology associated with atherosclerosis. Endothelial cells (ECs), macrophages, and smooth muscle cells have been demonstrated as the primary cell types participating in atherosclerosis [3]. The vascular endothelium is very important to respond to stimulation and to maintain vascular homeostasis. Endothelial dysfunction has been considered as the basis and initial step of many cardiovascular diseases, such as hypercholesterolemia, hypertension, and atherosclerosis. Thus, injury of ECs has been

considered as an atherosclerotic risk factor [3]. Oxidized low-density lipoprotein (ox-LDL) has been reported to be able to induce apoptosis of ECs and contribute to the progression of atherosclerosis [4]. Inhibition of ox-LDL-induced endothelial injury is an optional therapeutic strategy for vascular diseases.

Autophagy has been known as a conserved catabolism to sustain cellular homeostasis [5,6]. Autophagy plays critical roles in eliminating dysfunctional or surplus proteins and damaged intracellular organelles [7]. Accumulating evidence suggests that autophagy is implicated in ECs response to pathophysiological stimuli [8]. Moreover, autophagy is found to be activated in advanced atherosclerotic plaques by oxidized lipids, inflammation, and metabolic stress conditions [9]. A previous study also revealed that ox-LDL could induce vascular ECs autophagy and apoptosis in ApoE<sup>-/-</sup> mice [10].

Flavonoids have been described to possess beneficial effects on cardiovascular diseases by improving endothelial function [11]. Kaempferol is an important active member of dietary flavonoids discovered in many kinds of fruits and vegetables. Numerous studies have shown that kaempferol possesses broad bioactivities, including antioxidant, anti-inflammatory, antiapoptotic, and anticancer effect [12]. It has been proved that consumption of foods with abundant kaempferol lowers the risk of cardiovascular diseases [13]. Moreover, Pi et al. demonstrated that kaempferol effectively protected human umbilical vein

**Abbreviations:** ox-LDL, oxidized low-density lipoprotein; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; FCM, flow cytometry method; RAP, rapamycin.

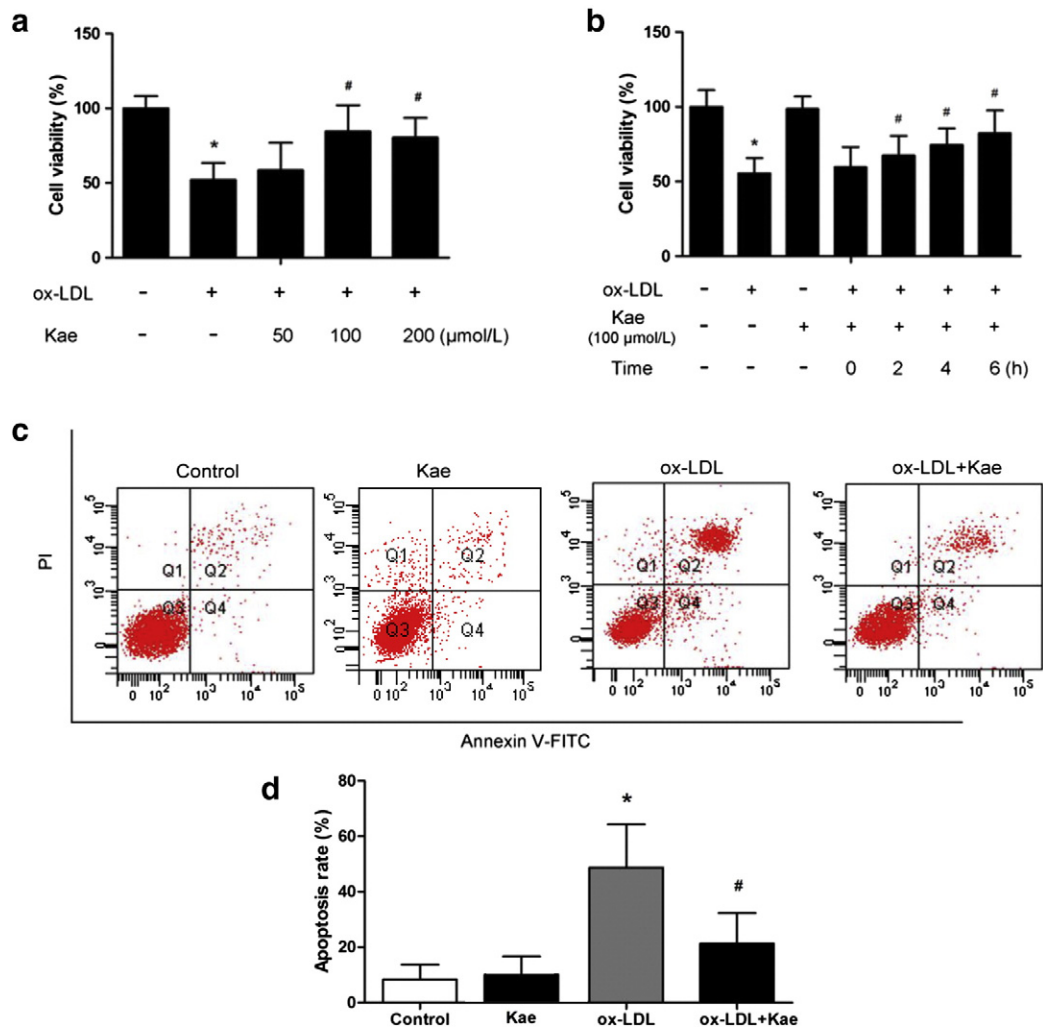
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**Fig. 1.** Effect of kaempferol on cell viability and apoptosis of ox-LDL-induced HUVECs. HUVECs were cultured with or without the pretreatment of kaempferol and then stimulated with ox-LDL for 12 h. The cell viability and apoptosis were detected by MTT and FCM, respectively. (a) Effect of kaempferol with different concentrations on the cell viability of ECs. (b) Effect of kaempferol with different pretreatment time on the cell viability of ECs. (c) Cell apoptosis of ECs with different treatment. (d) Quantitative analysis of cell apoptosis. \* $P < .05$  vs. control group, # $P < .05$  vs. ox-LDL group. Data are represented as mean  $\pm$  SD of three dependent experiments.

endothelial cells (HUVECs) against oxidative stress induced by ox-LDL [14]. Xiao et al. reported that kaempferol suppressed reactive oxygen species production in mice aorta and in HUVECs [15]. All these indicated that kaempferol was capable of preventing atherosclerotic vascular disease. However, the detailed molecular mechanism of kaempferol exerting protective effects on atherosclerosis remains far from being elucidated.

The present study indicated that kaempferol prevented ox-LDL-induced apoptosis of HUVECs. Furthermore, the protective effect of kaempferol on ox-LDL-triggered endothelial damage was mediated by up-regulation of autophagy via inhibiting PI3K/Akt/mTOR pathway.

## 2. Material and methods

### 2.1. Cell culture and treatment

HUVECs were obtained from ATCC (CRL-1730, Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere. HUVECs were cultured with or without the pretreatment of kaempferol (Sigma, USA), followed by stimulation with ox-LDL (100 mg/L; Biomedical Technologies, MA, USA). To investigate the effect of kaempferol on PI3K/Akt/mTOR pathway, cells were

treated with LY294002 (10 μM; Cell Signaling Technology, USA), rapamycin (RAP, 100 nM; Sigma, USA), or insulin (200 nM; Sigma, USA).

### 2.2. MTT assay

HUVECs were seeded in 96-well plates ( $1 \times 10^5$ ) in triplicate. Then, HUVECs were preincubated with different concentrations of kaempferol (50, 100, 200 μM) for 6 h or pretreated with kaempferol (100 μM) for different times (0, 2, 4, 6 h) prior to stimulation with ox-LDL for 12 h. A total of 20 μl of MTT solution (5 mg/ml, Sigma, USA) was added to each well and incubated for 4 h at 37°C. After the supernatants were removed, 150 μl of dimethylsulfoxide (Sigma, USA) was added to dissolve the formazan crystals. The cell viability was determined by measuring optical density at 570 nm.

### 2.3. Flow cytometry assay

HUVECs were preincubated with kaempferol (100 μM) for 6 h before stimulation with ox-LDL for 12 h. Apoptotic rate was analyzed using Annexin V–PI Apoptosis Detection Kit (Abcam, Cambridge, UK) by the flow cytometry method (FCM). Briefly, HUVECs were collected 48 h after stimulation and suspended in 500 μl of binding buffer. Then the cells were incubated with Annexin V at room temperature for 10 min and stained by propidium iodide prior to flow cytometry analysis.

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