Pharmacological Reports
2011, 63, 1180–1188
ISSN 1734-1140

Protective effect of α-lipoic acid on oxidized low density lipoprotein-induced human umbilical vein endothelial cell injury

Yan-Xia Liu, Guo-Zhu Han, Tao Wu, Peng Liu, Qin Zhou, Ke-Xin Liu, Hui-Jun Sun

Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, 9 West Section, Lvshun South Road, Lvshunkou District, Dalian 116044, Liaoning Province, China

Correspondence: Hui-Jun Sun, e-mail: sunhuijun@hotmail.com

Abstract:
The present study investigated the effect and possible mechanisms of α-lipoic acid (LA) in preventing endothelial cell injury induced by oxidized low-density lipoprotein (oxLDL). A model of human umbilical vein endothelial cell (HUVEC) injury was established by incubating the HUVECs with 200 μg/ml oxLDL. HUVECs were pre-treated with 0.1, 0.2 or 0.5 mmol/l of LA in the presence of oxLDL for 24 h. Apoptosis and cellular surface ceramide content were investigated separately by flow cytometry and by LC-MS/MS. LOX-1, Bcl-2 and CRP protein expression levels were evaluated by western blotting. LOX-1 mRNA expression was evaluated by RT-PCR assay. The results showed that oxLDL induced cytotoxicity in both concentration-dependent and time-dependent manners. LA boosted the cell survival rate and significantly reduced the content of MDA and lactate dehydrogenase (LDH) leakage. Apoptotic rates were significantly reduced by the addition of LA compared to oxLDL group. LA might also have inhibited ceramide generation induced by oxLDL in a dose-dependent manner. Furthermore, LA down-regulated LOX-1 protein and mRNA expression and up-regulated Bcl-2 protein expression levels in a dose-dependent manner. Expression of CRP protein was weak and undetectable. These results suggested that LA exhibited cytoprotective effects against oxLDL by decreasing apoptotic rates and decreasing cellular surface ceramide content, two effects that are related to decreased LOX-1 expression, and also by stimulating the expression of Bcl-2 protein. The cytoprotective effects are not thought to be due to inhibited C-reactive protein (CRP) protein expression in HUVECs.

Key words:
oxidized low density lipoprotein, α-lipoic acid, HUVEC, apoptosis

Introduction

Oxidative stress, a main risk factor for vascular endothelial cell apoptosis, is implicated in the pathogenesis of cardiovascular disorders. Oxidized low-density lipoprotein (oxLDL) plays a crucial role in the initiation and progression of atherosclerosis (AS). It is involved in the very early critical steps of atherogenesis, such as endothelial injury, expression of adhesion molecules, and leukocyte recruitment and retention [2, 25]. oxLDL is harmful to arterial cell types because it induces inflammatory cytokines and chemotactic factors overexpression [18]. Therefore, the search for protective factors for endothelial cells to prevent oxidative damage has become the focus of AS prevention. During the previous decade, it has been well established that the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) mediates

the uptake of oxLDL and plays a role in early AS [29]. When oxLDL binds to LOX-1 it rapidly stimulates an excess in ROS generation, contributing to endothelial dysfunction [9, 24]. α-Lipoic acid (6,8-thioctic acid, LA) is a sulfur-containing coenzyme required for the mitochondrial dehydrogenase reactions leading to ATP formation [6, 21, 23]. LA and the reduced form, dihydrolipoate, are potent natural antioxidants in both fat- and water-soluble media [21, 23]. Marangon et al. proved that LA functioned as an antioxidant by showing that LA supplementation in humans decreased plasma LDL-oxidation and urinary isoprostanes [20]. However, the cytoprotective effect of LA on oxLDL-induced vascular EC injury has not been explored. Moreover, less information has been available on the mechanism of anti-apoptosis effects of LA. In the present study, we investigated whether LA can protect against oxLDL-induced human umbilical vein endothelial cell (HUVEC) injury. We also investigated possible molecular mechanisms of LA's protective effect.

Materials and Methods

Reagents

RPMI-1640 was purchased from Gibco-BRL Company (GIBCO, USA). α-Lipoic acid was purchased from Sigma. Monoclonal anti-LOX-1, anti-Bcl-2, anti-CRP, and polyclonal rabbit anti-goat actin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-IgG was purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (USA). An annexin V-FITC apoptosis detection kit was purchased from Biosea Biotechnology (Beijing, China). Further specifications of other reagents are provided where mentioned.

Cell culture

HUVECs were incubated at 5% CO_2/37°C in RPMI Medium 1640 that was supplemented with 10% calf serum and penicillin (100 U)/streptomycin (100 μg/ml). The cells were examined under an invert microscope. When the cells were ~85% confluent, the culture medium was changed. The cells were divided into the following three groups: a control group, in which cells were incubated in RPMI Medium 1640; an oxLDL or native LDL group, in which cells were incubated for 24 h or 48 h with oxLDL (10–300 μg/ml) or native LDL (180 μg/ml); and an oxLDL + LA group, in which cells were pretreated with LA (0.1–0.5 mM) prior to incubation with oxLDL (180 μg/ml) for 24 h.

LDL preparation

Native LDL (optical density: 1.019–1.063 g/ml) was separated from the plasma of normal healthy volunteers by discontinuous density-gradient ultracentrifugation [30]. Blood was collected in tubes containing 1 mg/ml EDTA and was immediately centrifuged at 1750 × g at 4°C. Next, the prepared LDL was divided into two parts. One part was dialyzed for 36 h at 4°C against saline with phosphate buffered saline (PBS) which contained 0.01% EDTA. The other part was dialyzed overnight at 4°C against PBS to remove the EDTA and saline, which had oxidized. LDL and oxLDL protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit, which used bovine serum albumin as the standard and was expressed as micrograms per milliliter of solution [3]. LDL samples were stored at 4°C in the dark and were freshly prepared every 2 weeks.

LDL oxidation

Before the oxidation study, LDL was diluted in PBS to 1 mg protein/ml. Oxidation of LDL was carried out at 37°C under air in a water bath. LDL was incubated for 24 h at 37°C with freshly prepared 10 μmol/l $CuSO_4$. Oxidation was terminated by adding 10 μmol/l EDTA. To remove the $CuSO_4$, oxLDL was dialyzed overnight at 4°C against PBS containing 200 μmol/l EDTA. LDL oxidation was also determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) and the amount of lipid peroxides [1]. In this study, 180 μg/ml oxLDL-induced endothelial cell injury for 24 h was chosen as the injury model.

Cell viability assay

Cell viability was assessed by a mitochondrial tetrazolium assay (MTT) in the HUVECs [22]. Confluent cells were treated with various concentrations of LA or vehicle for 48 h. The HUVECs were incubated for 4 h at

37°C in fresh serum-free RPMI-1640 medium, which contained MTT at a final concentration of 0.5 mg/ml. Hundred microliters of acidic isopropyl alcohol was then added to each well for the overnight period. The absorbance of each well was measured by a Microplate reader (ThermoMultiskan Ascent, Finland) at λ = 570 nm with a referenced wavelength of 620 nm. Normal cells were used as the control group, and the cell viability of the control group was assumed to be 100%. The cell survival rate was calculated with the following formula [Eq. (1)]:

$$\text{Cell survival rate (\%)} = \frac{OD_{OX-LDL}}{OD_{Blank}} \times 100\% \quad (1)$$

Measurements of malondialdehyde (MDA)

To measure lipid peroxidation, MDA was measured by an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). First, the HUVECs were incubated with various concentrations of oxLDL for 24 h in the presence or absence of three different concentration of LA, 100 µl of supernatants were obtained. Then, the content of MDA was measured by the assay kit, with absorbance set at λ = 532 nm. The MDA contents were expressed as nmol/ml.

Measurements of lactate dehydrogenase (LDH)

To measure the extent of cell injury, the LDH activity was detected. At the end of the incubation period, the supernatants were collected. According to the LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the activity of LDH was detected at λ = 440 nm.

Apoptosis assays by annexin V flow cytometry

To detect apoptosis, the annexin V-binding capacity of treated cells was examined by flow cytometry (BD company, USA) using the ANNEXIN V-FITC APOPTOSIS DETECTION KIT I. The manufacturer's protocol was followed. After the HUVECs were incubated with various concentrations of oxLDL for 24 h in the presence or absence of three different concentrations of LA, cultured cells were collected and washed twice with cold PBS. Next, the cells were resuspended into 400 µl of binding buffer. Next, 5 µl annexin V-FITC and PI were added into 100 µl of cell suspension. After 15 min of incubation at 4°C in the dark, the cells were analyzed by flow cytometry.

Measurement of ceramide of cell membrane

Cells were harvested and washed twice with ice-cold PBS and were then resuspended in 220 µl of PBS. The samples were put in –20°C for 10 min and were sonicated for 20 s. After centrifugation at 3,000 × g for 15 min, the supernatant was discarded, and the precipitate, which contained the cell membrane, was resuspended in 220 µl of PBS. The samples were extracted with 1.33 ml of methanol:chloroform:water (1:2:1 by volume). The mixtures were vortexed for 5 min and then stood at room temperature for 30 min for phase separation. After centrifugation at 2,000 × g for 5 min, the lower organic layer was removed and dried under vacuum. The dried residue was dissolved in 100 µl of methanol for C16-ceramide detection by LC-MS/MS (API 3200; Applied Biosystems, Foster City, CA).

Western blot analysis

Total cell proteins were prepared from the HUVECs by western & IP Cell lysate (Biyuntian Biotechnology Research Institute, Shanghai, China). The protein content of cell lysates was quantified by a BCA kit. The HUVEC protein extracts, which contained equal amounts of total protein (15 µg), were applied to 10% SDS-PAGE gels. Using a Bio-Rad transfer blotting system at 300 mA for 4 h, they were then transferred to PVDF membranes (Millipore, Bedford, MA, USA) by wet-transfer. The membranes were blocked at room temperature for 2 h in 5% non-fat dry milk that was diluted with TBST (in mM: Tris-HCl 20, NaCl 150, pH 7.5, 0.1% Tween 20). The membranes were incubated overnight at 4°C with either anti-LOX-1 (1:500), anti-Bcl-2 (1:500) or anti-CRP (1:200). After three washes with PBS-T, the membranes were incubated at room temperature for 1 h with either a horseradish peroxidase-conjugated goat anti-mouse IgG or a goat anti-rabbit IgG (1:5000). The expression levels of LOX-1, Bcl-2, and CRP protein were determined by BeyoECL Plus regent (Biyuntian Biotechnology Research Institute, Shanghai, China). The relative density of the target bands was analyzed by a Gel Imaging Analyzer (USA). Incubation with polyclonal rabbit β-actin antibody was performed as the loading sample control.

Estimation of mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

Expression of the LOX-1 gene in the HUVECs was measured by a reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was extracted from the HUVECs with a TRIZOL reagent (Invitrogen). In the reverse transcriptase (RT) reactions, an equal amount of total RNA (500 ng) from the HUVEC samples was incubated for 3 min at 70°C and was subsequently reverse transcribed into cDNA, following the protocol of the kit (Takara, Dalian, China). cDNA was amplified using a primer pair specific to human LOX-1 (sense primer: 5'-CCTTGCTCGGAAGCTGAATG-3', antisense primer: 5'-CAGCGCCTCGGACTC TAAAT-3'). The PCR profile was completed in the following order to amplify a 377 bp product: 35 thermal cycles at 94°C for 40 s, 52°C for 30 s, and 72°C for 1 min. As an internal control, a 553 bp product of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense primer:5'-GTCTTCT GGGTGGCAGTGATG-3', antisense primer: 5'-ATGGTGAAGGTCGGTGTGAAC-3') was amplified. The PCR profile was as follows: 30 thermal cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The RT-PCR products were separated on 2% agarose gels and were visualized by ethidium bromide staining. Relative intensities of bands were analyzed by the Gel Imaging Analyzer and were expressed as ratios of the GAPDH mRNA band.

Results

Concentration and time-dependent cytotoxicity in HUVEC induced by oxLDL

Incubation with native LDL did not induce apoptosis, but oxLDL induced concentration-dependent and time-dependent cytotoxicity (Tab. 1). The cell survival rate was measured by a MTT assay after 24 h or 48 h exposure to oxLDL. The survival rates of the HUVECs were 28.1–35.5% and 15.1–15.5%, relatively, after they were incubated with 300 μg/ml oxLDL for 24 h or 48 h (Fig. 1A). After 48 h incubation, the content of MDA in the higher concentrations of the oxLDL groups was higher than the content in

Tab. 1. Cytotoxic effect of oxLDL in HUVECs for 24 h, 48 h, MTT assays (value of OD) ($\bar{x}$ SD, n = 3)

Groups	24 h	48 h
control	0.917 ± 0.0289	1.252 ± 0.0851
100 μg/ml oxLDL	0.737 ± 0.0523$^{\#}$	0.7435 ± 0.0516$^{\#\#}$
200 μg/ml oxLDL	0.507 ± 0.0099$^{\#\#}$**	0.291 ± 0.001$^{\#\#}$**
300 μg/ml oxLDL	0.264 ± 0.0071$^{\#\#}$**	0.192 ± 0.002$^{\#\#}$**

Note: $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* control group; ** $p < 0.01$ *vs.* 100 μg/ml oxLDL group

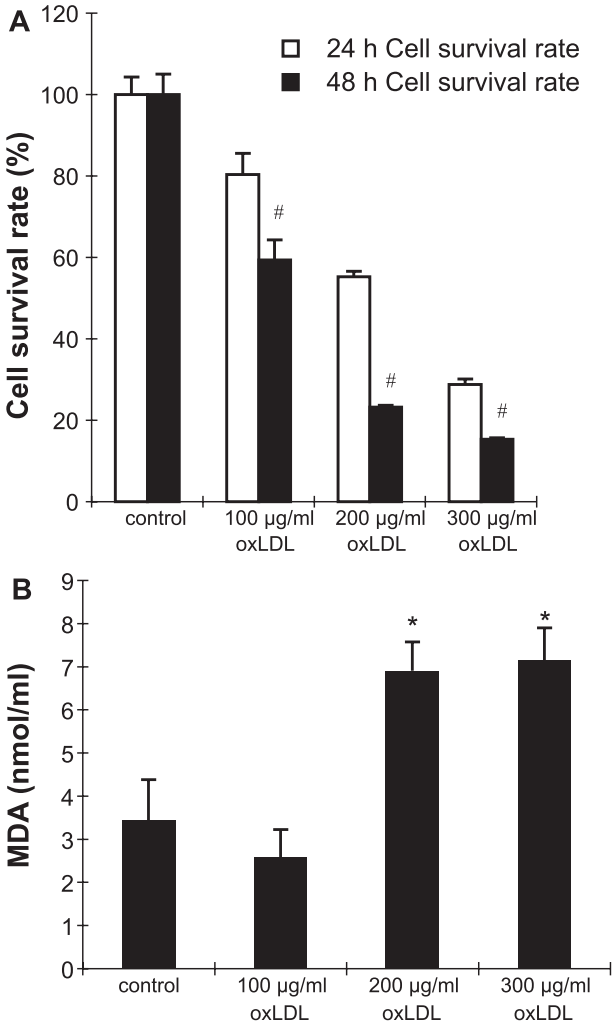

Fig. 1. Dose and time-dependent induction of cytotoxicity in HUVECs by oxLDL. (**A**) HUVECs (5×10^4 cells/ml) were treated with oxLDL at 100 μg/ml, 200 μg/ml and 300 μg/ml for 24 h and 48 h. At the end of the incubation time, the viability of the cells was evaluated by MTT assays. (**B**) Triplicate samples of supernatant were used to assay MDA. Each value represents the mean of three replicates, and the error bars represent SD. $^{\#}$ $p < 0.01$ *vs.* the same concentration group; * $p < 0.05$ *vs.* control group

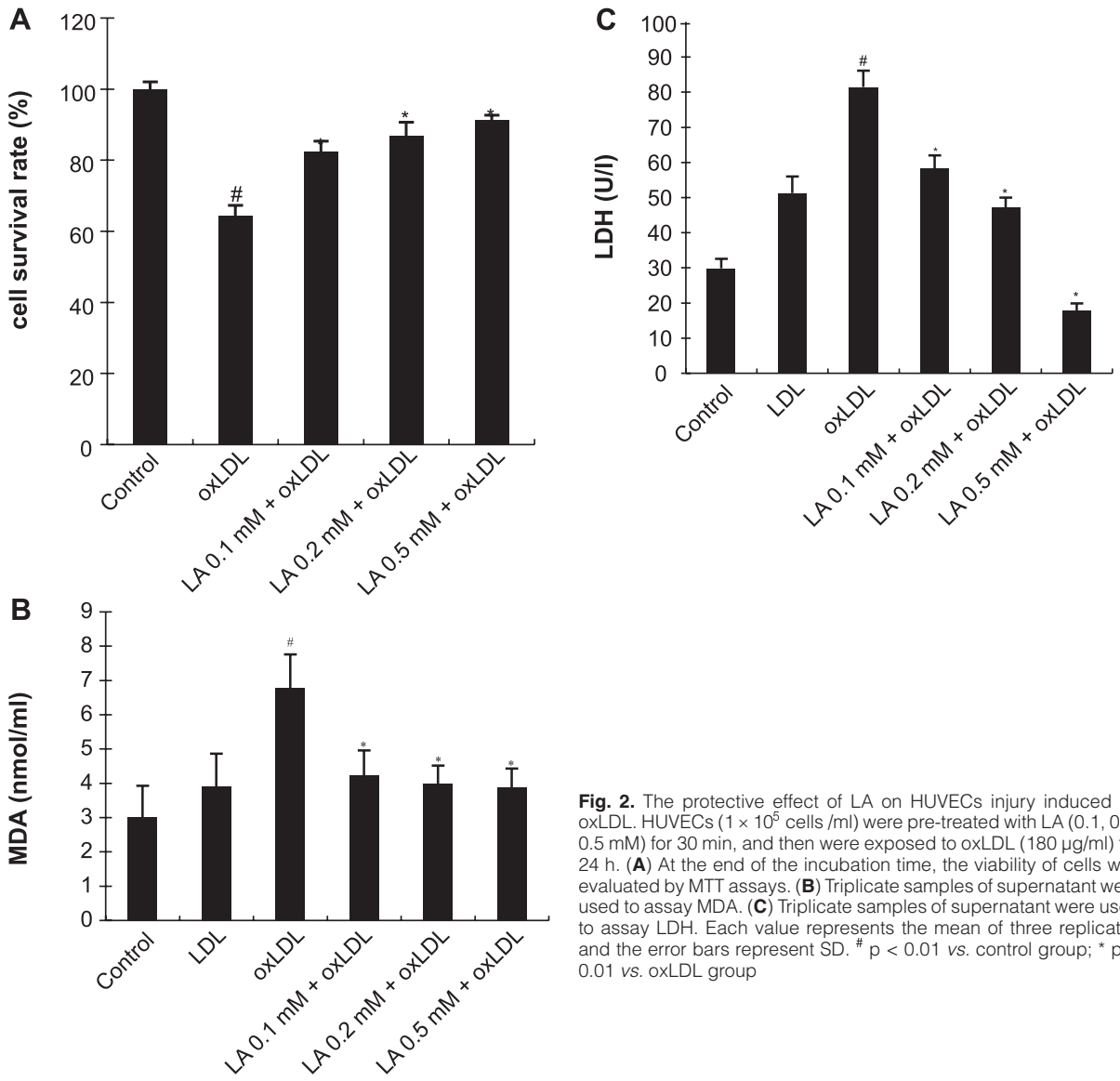

Fig. 2. The protective effect of LA on HUVECs injury induced by oxLDL. HUVECs (1×10^5 cells /ml) were pre-treated with LA (0.1, 0.2, 0.5 mM) for 30 min, and then were exposed to oxLDL (180 μg/ml) for 24 h. (**A**) At the end of the incubation time, the viability of cells was evaluated by MTT assays. (**B**) Triplicate samples of supernatant were used to assay MDA. (**C**) Triplicate samples of supernatant were used to assay LDH. Each value represents the mean of three replicates and the error bars represent SD. # $p < 0.01$ *vs.* control group; * $p < 0.01$ *vs.* oxLDL group

the other groups (Fig. 1B). The results showed that oxLDL caused cell injury in a concentration- and time-dependent manner.

Effect of LA on oxLDL-induced HUVEC injury

Incubation of HUVECs with oxLDL decreased cell viability. Decreased cell viability was indicated by inhibited MTT reduction and by augmented LDH release, compared to the n-LDL and vehicle control groups (Fig. 2A, 2C). This result was also accompanied by increased lipid peroxidation, which was indicated by elevated MDA content (Fig. 2B). All oxLDL effects were prevented by preincubation of the HUVECs with LA. Cell viability increased and the release percentage of LDH and MDA content decreased with increasing LA concentrations (Fig. 2A, 2B, 2C). A half mmol/l LA decreased the content of MDA by 40%, compared to the oxLDL group (Fig. 2B). Intriguingly, a similar result was obtained with LDH release (Fig. 2C).

Apoptosis assays by annexin V flow cytometry

The antiapoptotic effect of LA was measured 24 h after treatment by flow cytometry (Fig. 3A). Early apoptotic cells were identified by positive annexin V staining. Double-staining with annexin V and PI signified late apoptosis. PI-positive and annexin V-negative staining cells were necrotic cells. Annexin V-PI

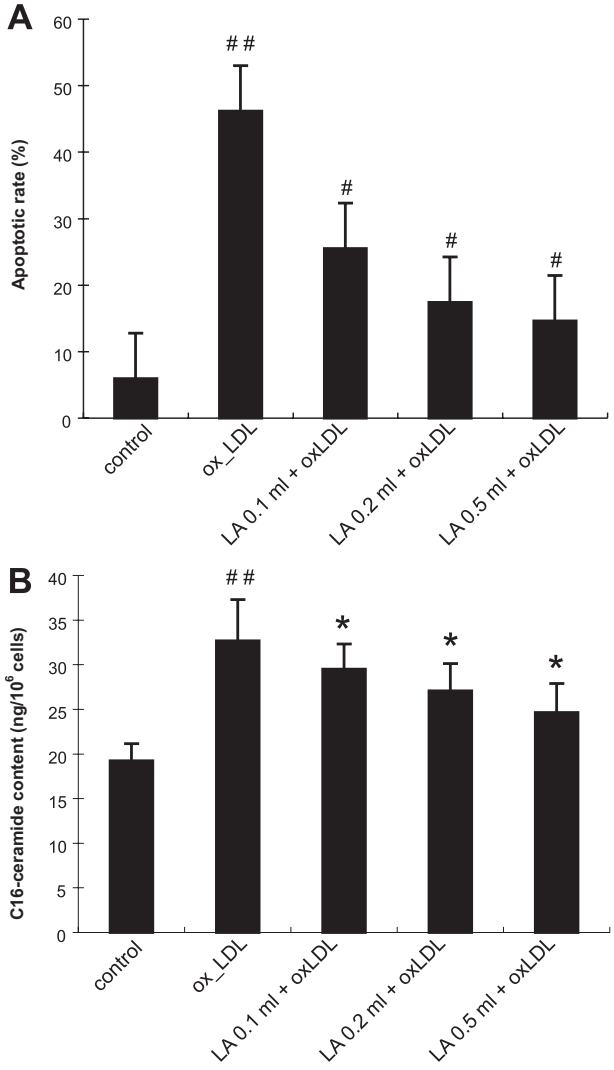

Fig. 3. Anti-apoptotic effect of LA against oxLDL-induced injury in HUVECs. Cells were cultured and collected as described in Materials and Methods. Treated cells were stained with annexin V-FITC and PI and were evaluated for apoptosis by flow cytometry (**A**). Cell membrane lipids were extracted and assayed for ceramide by LC-MS/MS assay (**B**). The bar graphs represent the mean ± SD of three independent experiments. Each experiment was performed in triplicate. ## $p < 0.01$ *vs.* control group; # $p < 0.01$ *vs.* oxLDL group (**A**); * $p < 0.05$ *vs.* oxLDL group (**B**)

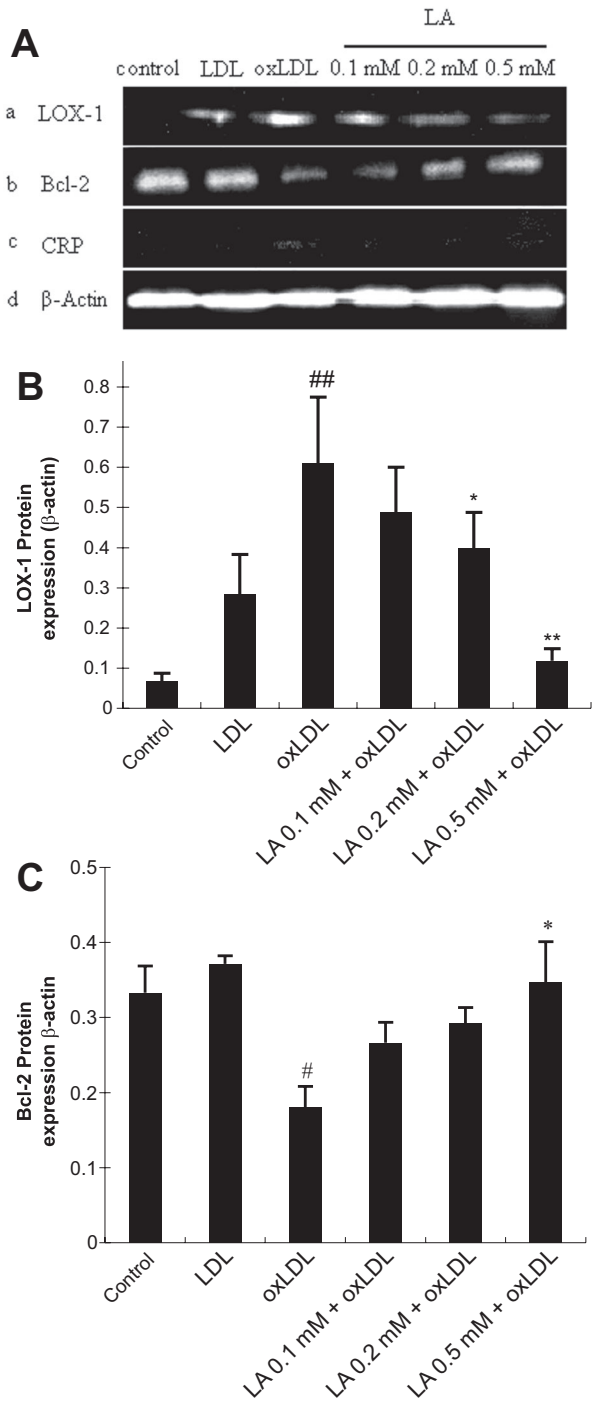

Fig. 4. Effect of LA on oxLDL-induced endothelial LOX-1, Bcl-2, and CRP protein expression in HUVECs. LA was pre-treated prior to the addition of 180 μg/ml oxLDL. At the end of the incubation period, total HUVECs protein extracts were electrophoresed on SDS-PAGE gels, followed by western blot analysis (**A**) with a primary antibody against LOX-1 (a) or Bcl-2 (b) or CRP (c). β-Actin protein was used as an internal control (d). LOX-1 and Bcl-2 protein expression levels were normalized to the levels of β-actin protein. The bar graphs represent the mean ± SD of three independent experiments (**B**, **C**). # $p < 0.05$, ## $p < 0.01$ *vs.* control group; * $p < 0.05$, ** $p < 0.01$ *vs.* oxLDL group

double-staining assays revealed that oxLDL significantly induced the apoptosis of the HUVECs (46.17 ± 1.62%), compared to the control cells (5.96 ± 1.33%) ($p < 0.01$). However, when the HUVECs were exposed to increasing concentrations of LA, a concomitant dose-dependent decrease in apoptotic rates, compared to the oxLDL group, was observed ($p < 0.01$). The apoptosis rates were 25.5 ± 2.46%, 17.42 ± 1.56%, and 14.63 ± 1.76%, respectively.

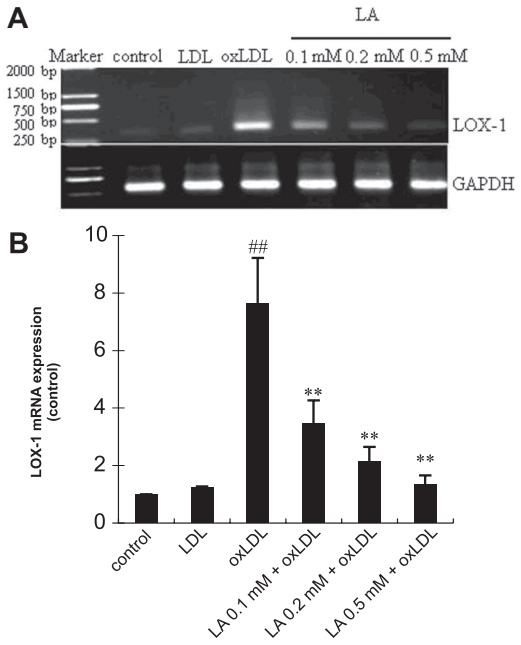

Fig. 5. Effect of LA on oxLDL-induced endothelial LOX-1 mRNA expression in HUVECs. LA was pretreated to HUVECs 30 min before oxLDL was added. (**A**) 24 h later, LOX-1 mRNA expression in the HUVECs was analyzed by RT-PCR. (**B**) LOX-1 mRNA levels were normalized to the levels of GAPDH mRNA. Data illustrated on the graph bar represent the mean ± SD of the experiment. Each value represents the mean of three replicates. ## p 0.001 *vs.* control group, ** p < 0.001 *vs.* oxLDL group

Measurement of ceramide of cell membrane

The lipid second messenger ceramide has been implicated in a number of cellular processes, including growth arrest and apoptosis [11]. Therefore, we studied whether ceramide was involved in oxLDL-induced apoptosis. We examined whether oxLDL affected cellular ceramide content by using a sensitive and specific LC-MS/MS technique. After 24 h of loading, oxLDL led to a significant increase in the content of cellular surface ceramide. This increase indicated that oxLDL had induced apoptosis by generating ceramide. As shown in Figure 3B, LA inhibited oxLDL-induced ceramide generation in a dose-dependent manner, suggesting that LA might mediate its antiapoptotic effects *via* inhibition of ceramide production.

Western blot analysis

Both *in vitro* and *in vivo* studies had shown that the uptake of oxLDL by endothelial cells occurs through a purely receptor pathway, the LOX-1 pathway. To explore whether LA exerted its protective action against oxLDL *via* an inhibition of LOX-1-dependent pathways, the HUVECs were analyzed with respect to LOX-1 protein in the absence or presence of oxLDL and LA. The high concentration of LA decreased the expression of LOX-1 by 86.273 ± 8.857%. The result showed that LA completely inhibited the effect of oxLDL, which could have increased the expression of LOX-1 (Fig. 4A, 4B).

To examine whether the protective effect of LA against oxLDL occurred because it inhibited apoptosis pathways, we also investigated changes in Bcl-2 expression in the HUVECs (Fig. 4A, 4C). Stimulation with oxLDL at 180 μg/ml for 24 h significantly decreased Bcl-2 protein expression, compared to the control group. When the cells were pre-treated with LA, Bcl-2 expression was markedly up-regulated, especially with 0.5 mmol/l LA.

Atherosclerosis is a chronic inflammatory process. C-reactive protein (CRP) is a characteristic marker of inflammation. We studied CRP expression in HUVECs. In all groups, CRP protein expression was weak and undetectable (Fig. 4A). As the CRP protein was not able to be activated by oxLDL in the HUVECs, we speculated that the effect of LA was not due to inhibition of CRP protein expression in HUVECs and that the CRP protein was not expressed by endothelial cells.

Expression of LOX-1 mRNA

As shown in Figure 5A, LOX-1 mRNA expression significantly increased by approximately 7.63 ± 1.59 fold, compared to the control group, in response to oxLDL. LA reduced the oxLDL-induced expression of LOX-1 mRNA in a significant concentration-dependent manner ($p < 0.001$) (Fig. 5B). CRP mRNA expression could not be detected in endothelial cells (data not shown).

Discussion

oxLDL-mediated injury to endothelial cells is crucial for endothelial dysfunction in early atherosclerosis and for atherosclerotic plaque rupture in advanced stages of atherosclerosis [27]. A model of *in vitro* oxLDL-induced endothelial cell injury has been applied to mimic the oxidative endothelial injury during

atherogenesis [7]. Therefore, in the present study, we investigated the protective effects of LA in a model of oxLDL-induced endothelial cell injury *in vitro*. HUVEC cultures were preincubated in the absence or presence of LA (0.1–0.5 mM) and were then incubated with oxLDL (180 μg/ml) for 24 h. Some previous reports have indicated that oxLDL could induce apoptosis in endothelial cells [10], and we have confirmed these prior observations in our study. The results showed that oxLDL impaired the cells, decreased cell viability and increased the release percentages of LDH and MDA content. To our knowledge, our study is the first demonstration that LA significantly reduced oxLDL-induced endothelial cell injury in a dose-dependent manner. In parallel with its protective action, LA could protect against cytotoxic effects of oxLDL, as assessed by cell viability, LDH and MDA release. Oxidative stress contributes to cellular injury and appears to be the common apoptotic mediator, most likely *via* lipid peroxidation [5]. Our results indicated that LA appeared to have the potential to reduce LDL oxidation and in turn to protect HUVECs against peroxidative toxicity (Fig. 1B).

The present study demonstrated that oxLDL produced a rapid apoptotic response in the HUVECs, accompanied by an elevation in ceramide levels. Increased ceramide levels conditioned the cells to commit apoptosis (Fig. 3A, 3B). The mechanism of action involved in the anti-apoptotic effect of LA might have been to decrease cellular surface ceramide content.

LOX-1 was originally identified as the major receptor for oxLDL in endothelial cells. LOX-1 plays a major role in the pathology of vascular diseases [16, 17]. LOX-1 mediates the uptake of oxLDL and plays a crucial role in early atherosclerosis [23, 26]. As shown in the present study, an approximately 10/8-fold expression of LOX-1 protein/mRNA was observed in the oxLDL group, compared to the control group. LA significantly decreased the increased expression of LOX-1 in a dose-dependent manner. These findings suggest that inhibition of LOX-1 expression might contribute to LA's role in anti-atherosclerosis.

Apoptosis of EC may destroy the vascular barrier integrity, expose smooth muscle cells to toxins, lipid, other vasoactive agents and neutrophils in the blood stream, and permit the extravasation of vascular inflammatory cells and proteins [8]. Hence, EC apoptosis has been implicated in numerous pathophysiological processes, such as angiogenesis, thrombosis, atherosclerosis, hypertension, diabetic vasculopathy and heart failure [14, 19]. Furthermore, in most cardiovascular diseases, oxidative stress plays a key role in EC damage (including apoptosis) [4]. It has been reported that Bcl-2 is an anti-apoptotic protein that can inhibit apoptosis induced by a mitochondria-dependent caspase-9 pathway [10, 15]. Overexpression of the Bcl-2 gene inhibits apoptosis in murine aortic endothelial cells [7, 12, 13]. Consistent with previous reports, we found that oxLDL induced significant endothelial cell injury and decreased Bcl-2 protein expression. In contrast, LA pretreatment dramatically reduced oxLDL-induced endothelial cell injury and upregulated oxLDL-induced decreased Bcl-2 protein expression. In LA-treated cells, the blockage of oxLDL's ability to decrease Bcl-2 protein expression provided evidence that LA's potent anti-apoptotic capacity might be related to the restoration of Bcl-2 protein expression.

CRP is a sensitive marker of inflammation. Under normal circumstances, CRP exists in the plasma in trace amounts. It significantly increases when the body experiences various forms of inflammation and trauma. AS is a chronic inflammatory disease. Inflammation penetrates in the whole course of the atherosclerosis. No sufficient evidence has been published to explain whether CRP independently induces the formation of atherosclerotic plaque. We assumed that ox-LDL, as a key factor in the early formation of atherosclerosis, could stimulate endothelial cells to express CRP. However, the results showed that oxLDL-pretreated HUVECs barely expressed CRP, providing evidence that CRP proteins found in the lesions were essentially derived from the circulation rather than synthesized de novo by vascular cells. This finding is consistent with our previous reports [28].

In conclusion, our results suggested that LA protected endothelial cells against oxLDL-induced injury. Also, this effect of LA was associated with mechanisms linked to LOX-1 blockade and anti-apoptotic Bcl-2 expression. Oxidative stress induces endothelial cell injury. As oxidative stress plays a key role in cardiovascular diseases, these effects of LA on endothelial cells could likely contribute to protection against atherosclerosis and other cardiovascular disorders.

Acknowledgments:
The authors thank Jiang-Lin Fan (Department of Pathology, University of Yamanashi, Japan) for his technical assistance. This study was supported by grants from the Science Foundation of Science & Technology Bureau of Liaoning Province (No. 20082156), the Provincial Key Laboratory for

Pharmacokinetics and Transport, Liaoning, China (No. LS2010053), and the Dalian Government (No. 2007J22JH012).

References:

1. Aviram M, Vaya J: Markers for low-density lipoprotein oxidation. Methods Enzymol, 2001, 335, 244–256.
2. Berliner JA, Heinecke JW: The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med, 1996, 20, 707–727.
3. Biffl WL, Moore EE, Moore FA, Barnett C: Nitric oxide reduces endothelial expression of intercellular adhesion molecule (ICAM-1). J Surg Res, 1996, 63, 328.
4. Boots AW, Haenen GR, Bast A: Health effects of quercetin: from antioxidant to nutraceutical. Eur J Pharmacol, 2008, 585, 325–337.
5. Buttke TM, Sandstrom PA: Oxidative stress as a mediator of apoptosis. Immunol Today, 1994, 15, 7–10.
6. Carreau JP: Biosynthesis of lipoic acid via unsaturated fatty acids. Methods Enzymol, 1979, 62, 152–158.
7. Chen J, Mehta JL, Haider N, Zhang X, Narula J, Li D: Role of caspases in ox-LDL-induced apoptotic cascade in human coronary artery endothelial cells. Circ Res, 2004, 94, 370–376.
8. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS et al.: Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood, 1998, 91, 3527–3561.
9. Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, Rigoni A et al.: Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-κB through an increased production of intracellular reactive oxygen species. J Biol Chem, 2000, 275, 12633–12638.
10. Escargueil-Blanc I, Meilhac O, Pieraggi M-T, Arnal J-F, Salvayre R, Negre-Salvayre A: Oxidized LDLs induce massive apoptosis of cultured human endothelial cells through a calcium-dependent pathway. Prevention by aurintricarboxylic acid. Arterioscler Thromb Vasc Biol, 1997, 17, 331–339.
11. Hannun YA, Luberto C: Ceramide in the eukaryotic stress response. Trends Cell Biol, 2000, 10, 73–78.
12. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ: Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell, 1993;75, 241–251.
13. Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T et al.: Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. Science, 1993, 262, 1274–1277.
14. Karsan A, Harlan JM: Modulation of endothelial cell apoptosis: mechanisms and pathophysiological roles. J Atheroscler Thromb, 1996, 3, 75–80.
15. Kondo S, Yin D, Aoki T, Takahashi JA, Morimura T, Takeuchi J: Bcl-2 gene prevents apoptosis of basic fibroblast growth factor-deprived murine aortic endothelial cells. Exp Cell Res, 1994, 213, 428–32.
16. Kume N, Kita T: Apoptosis of vascular cells by oxidized LDL: involvement of caspases and LOX-1 and its implication in atherosclerotic plaque rupture. Circ Res, 2004, 94, 269–70.
17. Li D, Mehta JL: Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. Circulation, 2000, 101, 2889–2895.
18. Li JM, Shah AM: Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. Am J Physiol Regul Integr Comp Physiol, 2004, 287, R1014–1030.
19. Liuzzo G, Giubilato G, Pinnelli M: T cells and cytokines in atherogenesis. Lupus, 2005, 14, 732–735.
20. Marangon K, Devaraj S, Tirosh O, Packer L, Jialal I: Comparison of the effect of α-lipoic acid and α-tocopherol supplementation on measures of oxidative stress. Free Radic Biol Med, 1999, 27, 1114–1121.
21. Moini H, Packer L, Saris NE: Antioxidant and prooxidant activities of α-lipoic acid and dihydrolipoic acid. Toxicol Appl Pharmacol, 2002, 182, 84–90.
22. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983, 65, 55–63.
23. Packer L, Witt EC, Tritschler HJ: Alpha-lipoic acid as a biological antioxidant. Free Radic Biol Med, 1995, 19, 227–250.
24. Ross R: Atherosclerosis-an inflammatory disease. New Engl J Med, 1999, 340, 115–126.
25. Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature, 1993, 362, 801–809.
26. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T et al.: An endothelial receptor for oxidized low-density lipoprotein. Nature, 1997, 386, 73–77.
27. Steinberg D: Lewis A. Conner Memorial Lecture: Oxidative modification of LDL and atherogenesis. Circulation, 1997, 95, 1062–1071.
28. Sun H, Koike T, Ichikawa T, Hatakeyama K, Shiomi M, Zhang B, Kitajima S et al.: C-reactive protein in atherosclerotic lesions: its origin and pathophysiological significance. Am J Pathol, 2005, 167, 1139–1148.
29. Taye A, Sawamura T, Morawietz H: Aldosterone augments LOX-1-mediated low-density lipoprotein uptake in human umbilical artery endothelial cells. Pharmacol Rep, 2010, 62, 311–318.
30. Wang CB, Feng YM, Zong YQ, Deng YZ, Feng ZC: Rapid isolation of large amount of plasma VLDL and LDL by a two step ultracentrifugation. J Tongji Med Univ, 1995, 15, 198–200.

Received: October 12, 2010; **in the revised form:** May 9, 2011; **accepted:** June 1, 2011.