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Isorhapontigenin and resveratrol suppress oxLDL-induced proliferation and activation of ERK1/2 mitogen-activated protein kinases of bovine aortic smooth muscle cells

Yinglin Liu, Gengtao Liu*

Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

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Abstract

The objective of our study was to compare the inhibitory effect of isorhapontigenin (ISO) and resveratrol, two natural antioxidants, on oxidized low-density lipoprotein (oxLDL)-induced proliferation of bovine aortic smooth muscle cells (BASMCs) and its relation to reactive oxygen species (ROS) generation and extracellular signal-regulated kinase 1/2 activation. The results showed that stimulation of oxLDL (50–150 μg/mL) for 48 hr induced a dose-dependent increase in cell number and incorporation of [^{3}H]thymidine into DNA of BASMCs. Western blot analysis demonstrated that oxLDL (150 μg/mL) stimulated an evident phosphorylation of p42/44 MAP kinases in BASMCs. Incubation of BASMCs with oxLDL induced significant increase in ROS detected by using an oxidant-sensitive fluorescent probe of 2′,7′-dichlorofluorescin diacetate. The level of H_2O_2 in the medium of cultured BASMCs also increased markedly. Preincubation of BASMCs with ISO and resveratrol significantly inhibited oxLDL-induced cell proliferation and incorporation of [^{3}H]thymidine, and the phosphorylation of p42/44 MAP kinases in BASMCs as well. Furthermore, preincubation of BASMCs with ISO and resveratrol attenuated oxLDL-induced increases in ROS and H_2O_2 levels. The results suggested that oxLDL-induced acute formation of ROS and subsequent activation of redox-sensitive extracellular signal-regulated kinase 1/2 MAPK pathways, which might be important for mitogenic signaling of oxLDL in vascular smooth muscle cells. The inhibitory effect of ISO and resveratrol on oxLDL-induced mitogenesis of BASMCs might be taken through blocking the generation of ROS and activation of the ERKs pathway.

Keywords: oxLDL; Aortic smooth muscle cells; Resveratrol; Isorhapontigenin; ERKs; Proliferation

1. Introduction

Low-density lipoproteins (LDLs) modified by oxidant are believed to play an important role in atherogenesis because of the close correlation between increased LDL levels in plasma and atherosclerosis risk [1]. Recent studies suggested that oxidized low-density lipoprotein (oxLDL) could be a mitogen in atherosclerosis as well as in lesion propagation, pathogenesis and proliferation of aortic smooth muscle cells (SMC) [2–4]. Therefore, the proliferation of vascular SMC initiated by oxLDL in the intima of arterial wall is considered as a critical event in the development of atherosclerotic plaque.

In eliciting their mitogenic effect, oxLDL targets a variety of molecular effectors including activation of mitogen-activated protein kinases (MAPKs, a superfamily of proline-directed serine/threonine protein kinases) [5–7]. The MAPKs comprise a large family of kinases that include ERK1 ($p44^{mapk}$)/ERK2 ($p42^{mapk}$), JNKs (also known as the stress-activated PKs), and p38 MAPKs [8]. Several research groups have demonstrated that extracellular signal-regulated kinases (ERKs) are important mediators of proliferation and exogenous oxidants-elicited activation of the ERKs pathway [9–11]. Annickal's studies indicated that different stimuli used reactive oxygen

* Corresponding author. Tel.: +86-10-6316-5178; fax: +86-10-6301-7757.
E-mail address: gtliu2002@yahoo.com (G. Liu).

Abbreviations: BASMCs, bovine aortic smooth muscle cells; SMC, smooth muscle cells; FBS, fetal bovine serum; DCF-DA, 2′,7′-dichlorofluorescin diacetate; ERK, extracellular signal-regulated kinase; H_2O_2, hydrogen peroxide; ISO, isorhapontigenin; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; MTT, thiazolyl blue; oxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species.

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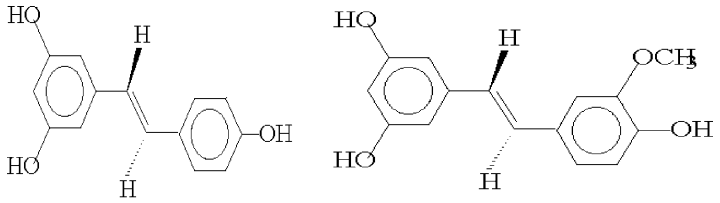

Fig. 1. Chemical structure of resveratrol and ISO.

species (ROS) as signaling messenger to activate transcription factors and induced gene expression [12]. Since oxLDL functioned as a prooxidant in atherogenesis, we speculated that there might exist an important link between ROS and oxLDL-induced cell proliferation.

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a polyphenol antioxidant and is abundant in grapes. Several recent studies have shown that resveratrol exhibited cardioprotective and chemopreventive effects [13,14]. Resveratrol may be an effective component for the reduced risk of coronary heart disease in humans who have consumed moderate grapes wine regularly. Although by what exact mechanism that resveratrol exerts its cardioprotective effect is not understood clearly, it is well known that resveratrol had the ability to inhibit oxidation of LDL, block platelet aggregation, and protect against oxidative injury to endothelial cells [15]. Isorhapontigenin (ISO, 3,4′,5-trihydroxy-3′-methoxy-stilbene) isolated from the Chinese herb *Belamcanda Chinesis* posessess similar chemical structure of resveratrol (shown below). Generally, similar chemical structural compound also has similar biological activity. Our previous studies showed that ISO also possessed antioxidative activity as evidence in inhibition of oxidation of human LDL and other prooxidant system *in vitro* [16,17].

However, there was no study dealing with the effect of ISO on proliferation of vascular SMC and its mechanism. Thus, the purpose of the present study was to compare the effect of ISO and resveratrol on oxLDL-induced proliferation of bovine aortic smooth muscle cell (BASMC) and its relation to ROS production and extracellular signal-regulated kinase 1/2 (ERK1/2) activation (Fig. 1).

2. Materials and methods

2.1. Materials

Isorhapontigenin and resveratrol were provided by Professor Mao Lin in the Institute of Materia Medica, Chinese Academy of Medical Sciences, their purity was over 98%. Healthy human plasma was obtained from Beijing friendship hospital. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), [^{3}H]thymidine(1 mCi/mL), were purchased from Sigma Chemical Co. Polyclonal anti-phospho-p42/44 MAP kinases (Thr202/Tyr204) antibodies were purchased from New England BioLabs. Polyclonal anti-ERK1/2 antibody and alkaline phosphatase goat anti-rabbit second antibody were from Santa Cruz Biotechnology. RPMI 1640 medium were obtained from Gibco-BRL. Fetal bovine serum (FBS) was purchased from Hyclone Ltd.

2.2. Preparation of LDL and oxLDL [18]

LDL was isolated from normal human plasma by sequential ultracentrifugation in the presence of 0.26 mM EDTA and stored at 4°. Just before the oxidation, LDL was separated from EDTA in 10 mM phosphate-buffered saline (PBS), and then the LDL (1.5 mg protein/mL) was exposed to 10 μM $CuSO_4$ for 18 hr at 37°. The extent of LDL oxidation was determined by evaluating the level of thiobarbituric acid-reactive substances as reported.

2.3. Cell culture

BASMCs were isolated from neonatal bovine aorta. Briefly, after striping coronary arterial endothelium, small pieces of media were carefully stripped from the vessel wall. Ten small strips (2 mm × 2 mm dimension) were placed in 100-mm diameter tissue culture dishes containing RPMI 1640 supplemented with 20% FBS in an atmosphere of 95% air, 5% CO_2 at 37°. Feed the tissues with 20% FBS RPMI 1640 medium every 2 days. Within 2 weeks, a large number of cells (SMCs) had migrated from the explants, SMC from the 100-mm dishes were subcultured in T-75 flasks and were identified according to morphological criteria and SMC between 5th and 10th passage were used in the experiments.

2.4. Assay of proliferation of BASMCs

BASMCs were routinely cultured in RPMI 1640 supplemented with 10% FBS. For measurement of cell growth, BASMCs were seeded in 10% FBS RPMI 1640 at 3000 cells per well (96-well plates) for 48 hr in a humidified CO_2 incubator at 37°. Cells were made quiescent by 48 hr incubation in RPMI 1640 medium containing 0.1% FBS. Then the quiescent cells were incubated with 0.1% FCS RPMI 1640 containing various concentrations of oxLDL for 48 hr, cell number was assayed by thiazolyl blue (MTT) test as described previously. The effect of ISO

and resveratrol on oxLDL-induced proliferation was studied under the same experimental conditions. Briefly, after preincubation with ISO and resveratrol (3–30 μM) for 1 hr at 37°, the BASMC were stimulated with oxLDL (150 μg/mL) for 48 hr. At the same time, the cytotoxicity of ISO and resveratrol was measured by incubating normal BASMCs with different concentrations of the test compounds without the stimulation of oxLDL.

2.5. Determination of [^{3}H]thymidine incorporation into DNA

BASMCs at 50% confluence in 96-well plates were made quiescent by 48 hr incubation in RPMI 1640 medium containing 0.1% FBS. After 48 hr incubation, the cells were incubated with 0.1% FCS RPMI 1640 containing various concentrations of oxLDL for 48 hr. At the end of 48 hr incubation, ^{3}H-TdR (1 μCi per well) was added into each well and incubated for an additional 6 hr. The cultured medium was aspirated, the cells were washed twice with PBS buffer and then were digested with 0.25% trypsin and 0.02% EDTA. The digested cells were collected on the glass fiber paper and put in scintillation liquid. The radioactivity of [^{3}H]thymidine into DNA was measured by the liquid scintillation counter.

The effect of ISO and resveratrol on [^{3}H]thymidine incorporation into DNA by oxLDL (150 μg/mL) was studied under the same experimental conditions as above.

2.6. Preparation of cellular lysates [5]

The confluent BASMCs in 100 mm petri dishes were starved with 0.1% FBS RPMI 1640 medium overnight and then stimulated with 150 μg/mL oxLDL for 5–30 min. At the end of stimulation, the cells were washed with ice-cold PBS and collected from lyses buffer containing 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 10 mM sodium fluoride, 1 mM Na_3VO_4, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 25 mM Tris–HCl, pH 7.4, 1% Triton X-100 and 0.5% Nonidet P-40 for 30 min at 4°. Then cell lysates were centrifuged at 8000 *g* for 30 min at 4°. The supernatant was collected and the protein concentration was determined by Lowry method. The effect of ISO and resveratrol on oxLDL-induced activation of p42/44 MAP kinases was performed by incubating the BASMCs with 30 μM ISO and resveratrol for 1 hr and then stimulated with 150 μg/mL oxLDL for 10 min. The cellular lysates were made under the same experimental conditions as above.

2.7. Western blot analysis of MAP kinases activity

Equal amount protein (100 μg) from each cell lyses sample prepared as above was separated on a 12% acrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The separated protein was then transferred to a PVDF membrane. The membrane was blocked for 2 hr at room temperature with 5% free-fat milk powder in TBS-T buffer (20 mM Tris, 500 mM NaCl, 0.05% Tween 20). The blot was then incubated with the anti-phospho-specific MAP kinases p42/44 antibody (1:500 dilution) at 4° overnight, followed by incubation for 2 hr with the secondary antibody (1:1000 dilution) of alkaline phosphatase goat anti-rabbit IgG. The immunoblot was visualized through NBT/BCIP system. The same blot was subsequently stripped and again probed with anti-ERK antibody as internal control. The protein bands were quantified by densitometry. The increases in MAP kinase activity were calculated as the ratio of phosphorylated MAP kinases to total MAP kinases.

2.8. Determination of intracellular reactive oxygen species (ROS) production through the oxidation of 2′,7′-dichlorofluorescin diacetate (DCF-DA)

DCF-DA is a dye that allows monitoring intracellular ROS production by laser confocal microscopy as described by Royall [19]. Confluent BASMCs in 35 mm tissue culture dish were incubated in RPMI 1640 containing oxLDL (150 μg/mL) for 1 hr. The cells were then washed with PBS and incubated with DCF-DA (10 μmol/L) for 10 min at 37°, the samples were immediately analyzed by laser confocal microscopy. The intensity of intracellular fluorescence of DCF indicated the production of intracellular ROS. The inhibition by ISO and resveratrol of oxLDL-induced ROS production was preformed by preincubation BASMCs with the test compounds (30 μM) for 1 hr and stimulated with oxLDL (150 μg/mL) for 1 hr as above.

2.9. Determination of hydrogen peroxide (H_2O_2) [20]

The quantification of H_2O_2 level was based on the horseradish peroxidase (HRPO)-dependent oxidation of phenol red, which was measured by its increased absorbance at 600 nm. One hundred microliters of free phenol red RPMI 1640 containing 150 μg/mL oxLDL or nLDL was added into each well of confluent BASMCs cultured in 96-well plate, and immediately 100 μL phenol red solution was added. The phenol red solution contained 140 mM NaCl, 10 mM potassium phosphate buffer, pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 200 unit/mL HRPO. The cells were incubated at 37° in a 5% CO_2 humidified atmosphere for 30 min. The reaction was stopped by adding 10 μL NaOH 1 mol/L per well and the plate was read at 600 nm on the Microplate reader (Bio-Rad Model 450).

2.10. Statistical analysis

The data were expressed as mean ± SD and Student's *t* test was used to analyze the statistical difference between groups.

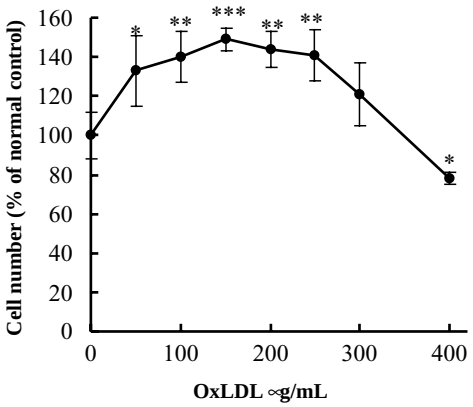

Fig. 2. Effect of oxLDL on growth and viability of BASMC. The cultured BASMCs were stimulated with oxLDL (50–400 μg/mL) for 48 hr, the cell number was measured by MTT test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with normal control.

3. Results

3.1. The stimulation of proliferation in bovine aortic smooth muscle cells (BASMCs) by oxLDL

The effect of oxLDL on proliferation of BASMCs was detected by MTT assay. As shown in Fig. 2, stimulation with oxLDL (150 μg/mL) for 48 hr resulted in approximate 50% increase in cell number, while the stimulated effect of oxLDL on BASMCs became weaker as the dose of oxLDL was over 200 μg/mL.

In order to investigate the effect of oxLDL on DNA synthesis in BASMCs, the incorporation of ^{3}H-TdR into DNA was measured after exposure of BASMCs to oxLDL for 48 hr. As shown in Fig. 3, the DNA biosynthesis of cultured BASMCs stimulated with oxLDL were enhanced in a concentration-dependent manner, and similar to the result of MTT assay, oxLDL 150 μg/mL induced maximum

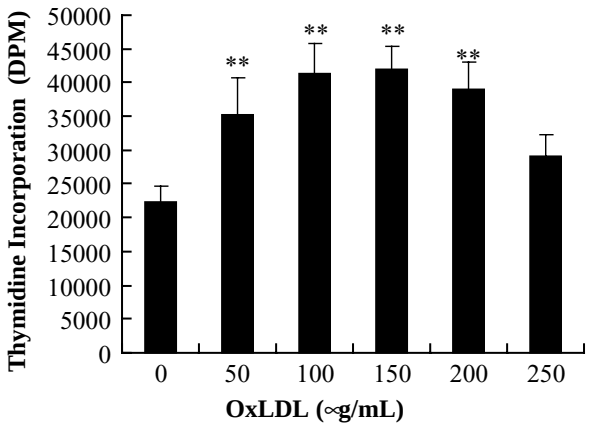

Fig. 3. Effect of oxLDL on DNA synthesis of BASMCs. The cultured BASMCs were stimulated with oxLDL (50–300 μg/mL) for 48 hr, 0.25 μCi of ^{3}H-TdR was then added to each well and incubated at 37° for 6 hr. The cells were harvested and the radioactivity of ^{3}H-TdR was detected by liquid scintillation counter. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with normal group.

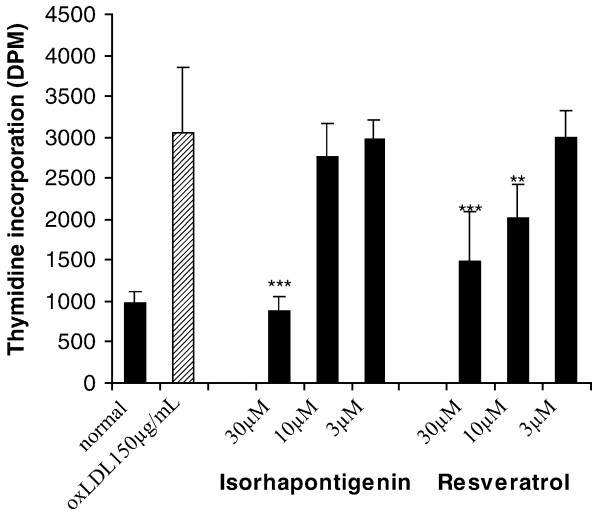

Fig. 4. Inhibitory effect of ISO and resveratrol on DNA synthesis of BASMCs induced by oxLDL. The cultured BASMCs were pretreated with ISO and resveratrol (3–30 μM), respectively, for 1 hr at 37°, and then stimulated with 150 μg/mL oxLDL for 48 hr. Before termination of the test, ^{3}H-TdR (0.25 μCi) was added to each well and incubated for 6 hr at 37°. The cells were harvested and the radioactivity of ^{3}H-TdR was counted by liquid scintillation counter. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with oxLDL group.

stimulation of DNA synthesis. When the concentration of oxLDL was over 150 μg/mL, the incorporation of ^{3}H-TdR [^{3}H] into the cells tended to decline.

3.2. Inhibition of oxLDL-induced proliferation of BASMCs by ISO and resveratrol

As shown in Figs. 4 and 5, preincubation of BASMCs with ISO and resveratrol (3–30 μM) significantly inhibited oxLDL-induced increases in DNA synthesis and cell number. The action of ISO at dose of 30 μM was slightly more potent than resveratrol, but no significant difference

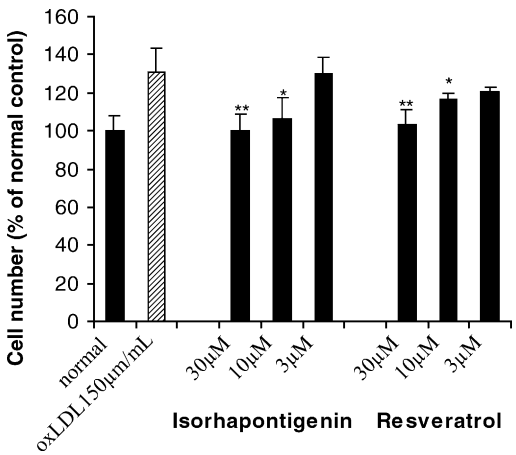

Fig. 5. Inhibitory effect of resveratrol and ISO on BASMC growth induced by oxLDL. The cultured BASMCs were pretreated with ISO and resveratrol (3–30 μM), respectively, at 37° for 1 hr, then stimulated with 150 μg/mL oxLDL for 48 h, the cell viability was measured by MTT method. Each group was compared with oxLDL group. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1
Effect of ISO and resveratrol on the growth and DNA synthesis of normal BASMCs

Group	Incorporation of ^{3}H-TdR (DPM)	Cell viability (%)
Normal control	9790 ± 1455	100 ± 10
ISO (30 μM)	8361 ± 1960*	89 ± 9*
Resveratrol (30 μM)	8874 ± 2370*	100 ± 4*

The cultured BASMCs were incubated with ISO and resveratrol (30 μM), respectively, for 48 hr. Before termination of the test, ^{3}H-TdR (0.25 μCi) was added to each well and incubated for 6 hr at 37°. The cells were harvested and the radioactivity of ^{3}H-TdR was counted by liquid scintillation counter. Cell viability was tested by MTT assay.

*$P > 0.05$ vs. normal control group.

was found between the two groups. The above concentrations of ISO and resveratrol were not shown to have any inhibitory effect on cell growth and DNA synthesis of normal BASMCs under the same experimental conditions (Table 1).

3.3. Effect of oxLDL on phosphorylation of MAP kinase isoforms p44/42 in bovine aortic smooth muscle cells

Activation of p44^{mapk} and p42^{mapk} was accompanied by phosphorylation of the Tyr204 residue. When BASMCs were stimulated with oxLDL (150 μg/mL) for different time, enhanced phosphorylation of the p44^{mapk} and p42^{mapk} occurred after 10-min stimulation and then declined (Fig. 6A). Densitometry detection of the blots revealed that oxLDL induced a 4-fold increase in both p44^{mapk} and p42^{mapk} isoforms as compared with control levels (Fig. 6B).

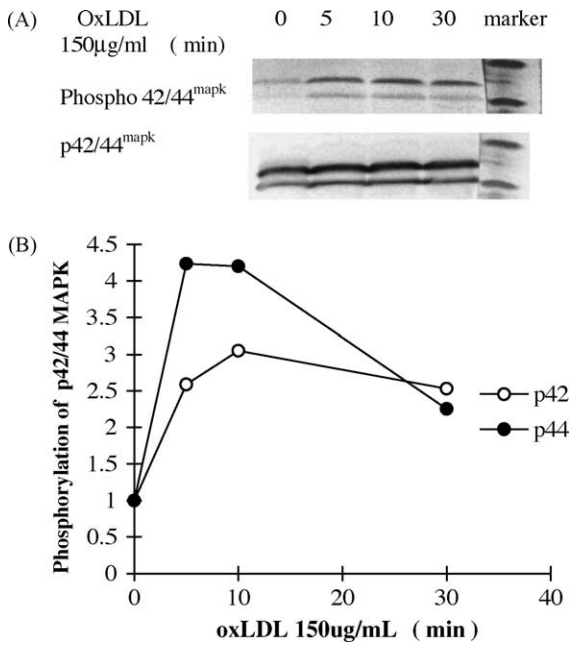

Fig. 6. Time course of oxLDL-induced phosphorylation of p42/44 MAPK in BASMCs. (A) Western blotting analysis showing phosphorylated p42 and p44. BASMCs were incubated with 150 μg/mL oxLDL for indicated time periods. Following stimulation with oxLDL, the cells were lysed and separated on a 12% acrylamide gel by SDS–PAGE electrophoresis and transferred to PVDF membranes. The immunoblot was obtained with specific anti-phospho-MAP kinase p42/44 antibody as described in Section 2. (B) Phosphorylation of MAP kinase was expressed as ratio to the control: the ratio was calculated by dividing phosphate-MAP kinase p42/44 to the total MAP kinase p42/44 in each group. Each group was compared with normal control group.

3.4. Inhibitory effect of isorhapontigenin and resveratrol on phosphorylation of MAP kinase isoforms p44/42 in BASMCs induced by oxLDL

For further investigating the effect of ISO and resveratrol on ERK1/2 signaling pathways, BASMCs were pretreated with ISO and resveratrol (30 μM), respectively. As a result, the oxLDL-stimulated phosphorylation of MAP kinase isoforms p44/42 in BASMCs was inhibited by ISO completely (Fig. 7). The inhibition of oxLDL-induced ERK1/2 phosphorylation by resveratrol was less

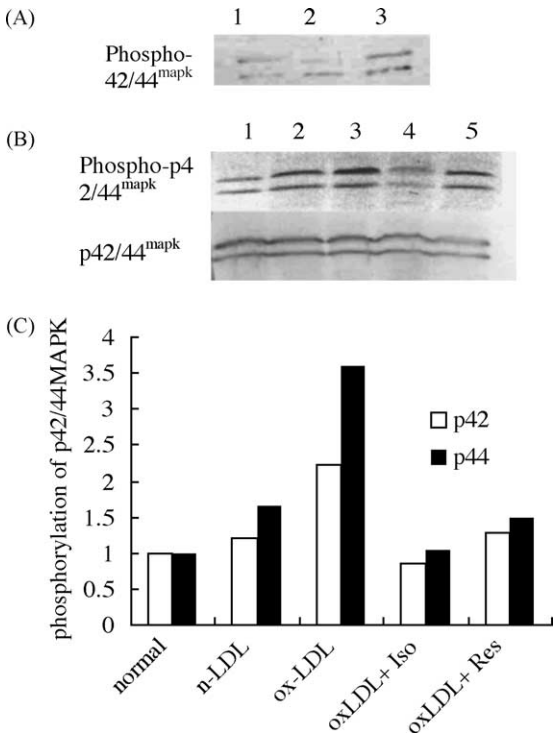

Fig. 7. Inhibitory effect of ISO and resveratrol on oxLDL-induced phosphorylation of p42/44 MAPK in BASMCs. (A) Effect of Iso and Res on the level of phosphorylated p42/44 MAPK in quiescent BASMCs. Growth arrested BASMCs were incubated with ISO and resveratrol (30 μM) for 60 min, respectively, and then the levels of phosphorylated p42/44 MAPK in whole cell lysis were determined by Western blot. 1, normal control; 2, ISO (30 μM) treated; 3, resveratrol (30 μM) treated. (B) Western blotting analysis showing phosphorylated p42 and p44 induced by oxLDL. BASMCs were preincubated with ISO and resveratrol (30 μM) for 60 min at 37°, respectively. And then treated with oxLDL (150 μg/mL) for 10 min at 37°. 1, normal; 2, 150 μg/mL nLDL; 3, 150 μg/mL oxLDL; 4, 30 μM ISO + oxLDL; 5, 30 μM resveratrol + oxLDL. (C) Phosphorylation of MAPK was expressed as ratio to the control: the ratio was calculated by dividing phosphate-MAP kinase p42/44 to the total MAP kinase p42/44 in each group. Each group was compared with normal control group.

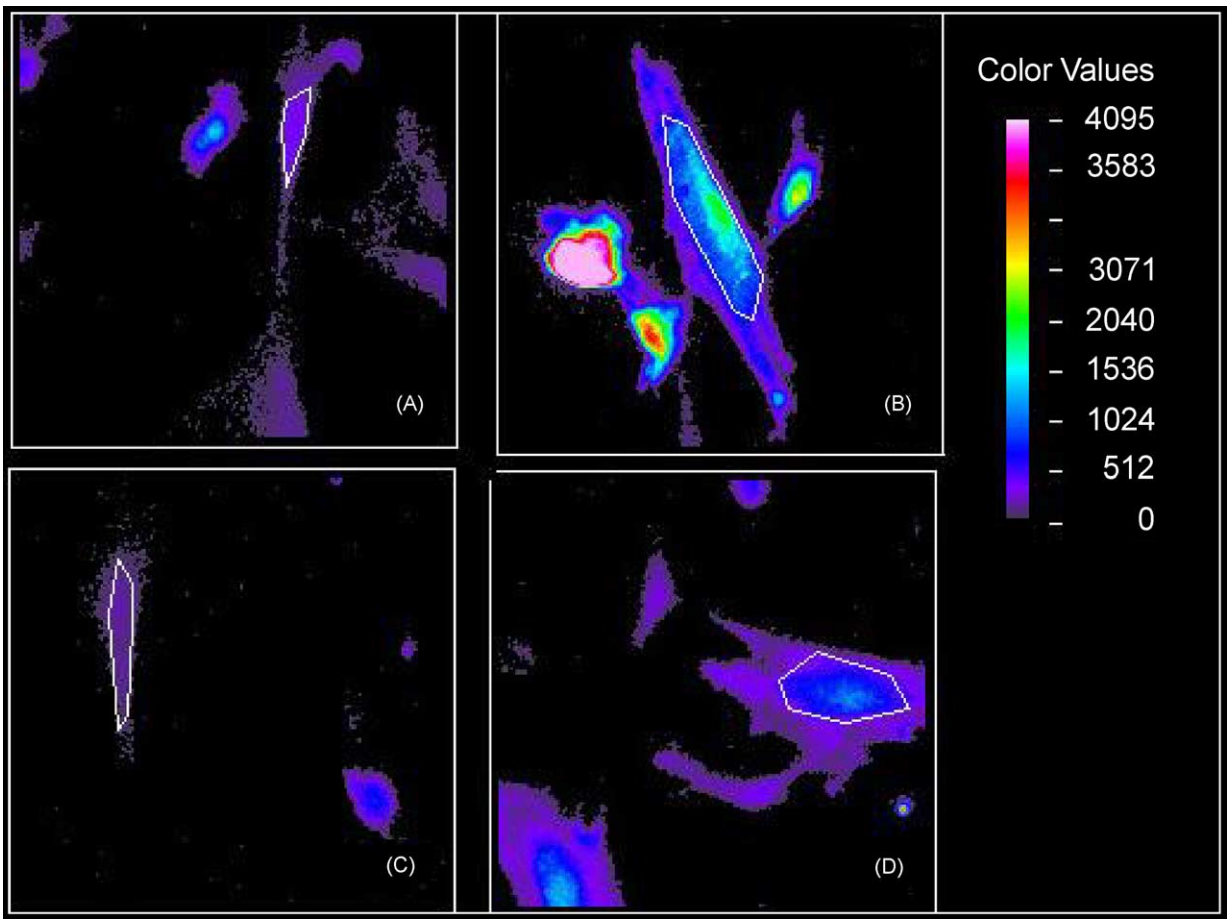

Fig. 8. Effect of ISO and resveratrol on oxLDL-induced intracellular free radicals formation in BASMCs. BASMCs were preincubated with ISO and resveratrol (30 μM), respectively, for 1 hr at 37°, and then stimulated with oxLDL (150 μg/mL) for 1 hr. Following stimulation with oxLDL, the cells were rinsed with PBS and incubated with DCF-DA (10 μmol/L) and observed by laser confocal microscopy. A, normal cells; B, 150 μg/mL oxLDL-treated cells; C, resveratrol (30 μM) pretreated cells; D, ISO (30 μM) pretreated cells.

pronounced but still evident. Furthermore, neither resveratrol nor Iso at concentration of 30 μM had a significant effect on basal mitogen-activated protein kinase (MAPK) activity in the quiescent BASMCs as shown in Fig. 7A. Presumably, the inhibition of ERK1/2 pathway may contribute to blockage of oxLDL-induced proliferation by resveratrol and ISO.

3.5. Inhibitory effect of isorhapontigenin and resveratrol on oxLDL-induced production of intracellular ROS in bovine aortic smooth muscle cells

The intracellular ROS production was monitored with DCF-DA dye under laser confocal microscope. As shown in the photographs of BASMCs under laser confocal microscopy (Fig. 8A–D), oxLDL (150 μg/mL) elicited a significant generation of ROS in BASMCs after 60-min stimulation (Fig. 8B) in comparison with normal control cells (Fig. 8A). ISO and resveratrol significantly reduced the generation of intracellular ROS stimulated by oxLDL (Fig. 8C and D).

Table 2
Inhibitory effect of ISO and resveratrol on oxLDL-induced generation of H_2O_2 in BASMCs

Group	H_2O_2 medium (nmol/mL)
Normal control	0.92 ± 1.18
nLDL (150 μg/mL)	0.88 ± 1.07
oxLDL (150 μg/mL)	13.38 ± 2.63
ISO (μM)	
30	2.56 ± 1.81**
10	5.48 ± 3.81**
3	8.78 ± 4.82*
Resveratrol (μM)	
30	2.35 ± 2.77**
10	8.24 ± 0.69**
3	12.47 ± 1.86

BASMCs were pretreated with ISO or resveratrol, respectively, for 1 hr, and then stimulated with 150 μg/mL oxLDL for 30 min at 37°. The production of H_2O_2 was measured by method of the HRPO-dependent oxidation of phenol red as described in Section 2. Each group was compared with oxLDL control groups. $x \pm SD$, $N = 8$.

*$P < 0.05$ vs. oxLDL group.

**$P < 0.01$ vs. oxLDL group.

3.6. Inhibitory effect of isorhapontigenin and resveratrol on the production of H_2O_2 in bovine aortic smooth muscle cells induced by oxLDL

The results in Table 2 indicated that incubation of BASMCs with oxLDL (150 μg/mL) for 30 min induced approximate 14-folds increase in H_2O_2 level in the cultural medium. In contrast with oxLDL, nLDL showed no any induction of H_2O_2 generation. Preincubation of the cells with ISO and resveratrol markedly inhibited the generation of H_2O_2.

4. Discussion

Increased proliferation of vascular SMC is a critical determinant of atherosclerosis, and oxLDL is a pivotal stimulus in the mitogenesis of vascular SMC [7]. As expected, our study also showed that oxLDL elicited significant proliferation response of BASMCs. oxLDL at low concentrations (50–150 μg/mL) increased cell number and DNA biosynthesis in a dose-dependent manner, and oxLDL at the dose of 150 μg/mL induced maximum increase in cell DNA synthesis by 180%. When the concentration of oxLDL was over 150 μg/mL, its stimulating effect on the incorporation of [^{3}H]thymidine into the cells tended to decline. The potency of oxLDL-induced proliferation in BASMCs in the present study was weaker than that reported by other researchers [3,4,7,21]. However, since there is potentially a continuous spectrum of degrees of oxidation and a great deal of molecular heterogeneity in what we call ''oxidized LDL,'' even if oxidative conditions are controlled as precisely as possible, the product will still vary from experiment to experiment depending upon the composition of the starting LDL [1]. More recent studies suggested that experiments to quantify oxLDL-induced increases in cell number were hindered by the counterbalance of proliferation and inhibitory toxic lipids [2].

oxLDL carries dozens of potentially bioactive lipids that are not appreciably present on native LDL. There have been several attempts to identify the components of oxLDL such as PAF-like lipids and oxidized phospholipids responsible for inducing proliferation. LysoPC and PAF-like lipids are not the only constituents of oxLDL linked to its proliferative activity. 4-Hydroxy-2-nonenal is a known constituent of oxLDL and has been shown to stimulate cell growth in rat SMC. In addition, certain oxidation products of unsaturated free fatty acids have been shown to stimulate mitogenic signaling pathways in SMCs, including hydroperoxyoctadecadienoic and hydroperoxyeicosatetraenoic acids [21–24]. These multiple lipids with diverse actions could interact with specific intracellular signaling pathways and exert selective control of cell growth.

Some studies observed that pretreatment of Vitamin E, superoxide oxidase and catalase with SMCs blunted the oxLDL- and lysoPC-induced SMC proliferation responses [25–27]. In our experiment, ISO and resveratrol markedly inhibit oxLDL-induced BASMCs proliferation. In addition, we observed the oxLDL-induced oxidative stress response through stimulating intracellular reactive oxygen generation as detected with the sensitive fluorescent dye DCFH-DA. ISO and resveratrol significantly attenuated the generation of intracellular ROS. H_2O_2 may also involve the mechanism of mitogenic effect of oxLDL. We observed that oxLDL induced remarkable generation of intracellular H_2O_2. A previous study showed that sublethal H_2O_2 exposure caused cell proliferation including in SMC, and H_2O_2 was also linked to bFGF release from human SMC [5,28]. The blocking of resveratrol and ISO on both mitogenesis and generation of ROS in BASMCs suggested that the anti-proliferative effect of both compound might be taken through blocking the generation of ROS in oxLDL-stimulated vascular SMCs.

A role of ROS, especially H_2O_2, in smooth muscle cell growth was further supported by the findings that exogenous H_2O_2 or chemical agents that can generate ROS are able to induce tyrosine phosphorylation of MAPKs and cell growth [29–31]. The results of our study indicated that oxLDL stimulated the phosphorylation of $MAPK^{p42/44}$ and increased smooth muscle cell proliferation markedly. Preincubation of BASMCs with resveratrol (30 μmol/L) or ISO (30 μmol/L) inhibited oxLDL-mediated activation of both $MAPK^{p42/44}$ and cell proliferation. These phenomena were accompanied by a concentration-dependent inhibition of oxLDL-induced generation of ROS in SMC. Many studies have demonstrated the ability of exogenous oxidants to activate the ERKs MAPK pathway. ERKs can be also activated by exogenous H_2O_2 and endogenously generated ROS in SMCs stimulated with growth factors [9,32,33]. The exact molecular target of the above effects is unclear. Some studies suggested that ROS-mediated ERK activation might be an upstream event at the level of growth factor receptors such as Src kinases and/or $p21^{Ras}$ [34,35]. Yamakawa *et al.* [36] reported that O_2^- and H_2O_2 could stimulate Ras GTP loading, which in turn stimulated the kinase cascade involving Raf, Mek-2 and ERK1/2 phosphorylation. The phosphorylated ERK further translocated into the nucleus from the cytoplasm, and then stimulated the expression of c-*fos* [37,38].

It has been reported that resveratrol itself exerted antiproliferative action via an inhibitory effect on MAPK activation in vascular smooth muscle [39]. However, our blot results showed that neither resveratrol nor Iso at concentration of 30 μM had a significant effect on basal MAPK activity in the untreated quiescent BASMCs. We inferred that the cells in our research were serum starved for 48 hr before the stimulation by oxLDL, hence the phosphorylation of ERKs in acquiesce untreated cells was very low, at the level of which the effect of resveratrol alone on ERKs activation in the absence of oxLDL was weak and could be ignored.

Although our research focused on the association of anti-proliferative action of resveratrol and ISO with ROS, resveratrol was postulated to inhibit the proliferation of SMCs by other mechanisms as well. Some authors reported that resveratrol arrested cell cycle progression at the S phase of mitosis [40,41]. Recent studies showed that ROS as signaling intermediates might promote the cell cycle progression. H_2O_2 may sustain the expression of cyclin D1 and D2 by reversibly inhibiting the ubiquitin-proteasome dependent degradation of cyclin D1 and D2 in Her14 fibroblasts [42]. Some studies also founded that p21waf1/cip1 expression could be regulated by intracellular redox conditions [43,44]. It will be interesting to know the effect of resveratrol and ISO on oxLDL-induced expression of cell cycle-related proteins in SMCs as well as the role of ROS in such expression.

Both ISO and resveratrol had comparable ability to inhibit the generation of ROS stimulated by oxLDL. Our study indicated that redox-sensitive ERKs activation played a crucial role in oxLDL-stimulated proliferation of vascular SMCs, which was consistent with other reports [21,22,25,27]. However, our study also showed discrepancy that ISO was more potent than resveratrol to inhibit the oxLDL-induced phosphorylation of ERKs and proliferation in vascular SMCs. We considered that there might be another mechanism beyond antioxidative activity of ISO contributing to such inhibition of mitogenesis. However, more studies are needed before definitive conclusion can be made regarding the mechanisms of anti-proliferation by ISO. In a short, the present study implied that the inhibitory action of resveratrol against oxLDL-mediated proliferation of vascular SMCs would contribute to its cardiovascular protection. The down regulation of $MAPK^{p42/44}$ and the inhibition of ROS generation by resveratrol and ISO in oxLDL-stimulated vascular SMCs could explain, at least in part, their anti-proliferation activity.

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