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Ginkgolide B Suppresses Intercellular Adhesion Molecule-1 Expression via Blocking Nuclear Factor-κB Activation in Human Vascular Endothelial Cells Stimulated by Oxidized Low-Density Lipoprotein

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Abstract. Atherosclerosis is a complex inflammatory arterial disease. Oxidized low-density lipoprotein (ox-LDL) is directly associated with chronic vascular inflammation. In the current study, we tested the hypothesis that ginkgolide B, a component of traditional Chinese herbal medicine for heart disorder, may affect ox-LDL–induced inflammatory responses in human umbilical vein endothelial cells (HUVECs). The results showed that the ox-LDL treatment caused a significantly increase in the expression of intercellular adhesion molecule-1 (ICAM-1) in HUVECs, which was associated with a dramatic augmentation in phosphorylation of IκB and relocation of nuclear factor-κB (NF-κB) into the nuclei. Interestingly, the ox-LDL–induced ICAM-1 expression and NF-κB relocation could be attenuated by addition of ginkgolide B. Moreover, ginkgolide B significantly reduces ox-LDL–induced generation of reactive oxygen species (ROS). In conclusion, ginkgolide B may decrease inflammatory responses induced by ox-LDL via blocking NF-κB signaling and inhibiting ROS generation in HUVECs.

Keywords: ginkgolide B, intercellular adhesion molecule-1 (ICAM-1),
oxidized low-density lipoprotein (LDL), nuclear factor-κB (NF-κB), endothelial cell

Introduction

Ginkgo biloba has been used as a herb in traditional Chinese medicine for thousands of years. *Ginkgo biloba* extracts contain flavonoid and terpenoid substances. Flavonoids may serve as free radical scavengers, especially for oxygen-derived free radicals, such as OH·, O^{2-}, RO·, and ROO·, and neutralize ferryl ion–induced peroxidation (1, 2). The terpenoid fraction contains ginkgolide and bilobalide and functions as an antagonist of platelet-activating factor. Moreover, it has been demonstrated that terpenoid mediated processes of platelet aggregation, arterial thrombosis, acute inflammation, allergic reaction, and cardiovascular insufficiency (3 – 5). Ginkgolides in the terpeniod extracts can be divided into isotypes A, B, C, M, and J, among which ginkgolide B ($C_{20}H_{24}O_{10}$) has the highest biological activity. Previous investigations have suggested that ginkgolide B was a specific antagonist for platelet-activating factor (PAF) receptor and could prevent PAF-induced ischemia-like cellular damage (6, 7). Furthermore, *Ginkgo biloba* extracts are routinely prescribed as medicine for age-related diseases such as memory disorders, atherosclerosis, Alzheimer's disease, ischemic heart disease, cerebral infarction, and nerve degeneration diseases (8, 9). The beneficial effects of ginkgo biloba extracts are beneficial and they may exert therapeutic effects on atherosclerosis by serving as anti-oxidative and free radical–scavenging activities (10).

Atherosclerosis is a slowly progressing inflammatory disease of the medium- and large-sized arteries, involving interactions between endothelial cells, vascular smooth muscle cells, macrophages, platelets, and cytokines. Atherosclerosis is the major cause of cardiovascular diseases and is responsible for most of deaths in the senior population.

Oxidized low-density lipoproteins (ox-LDL) have been demonstrated to induce multiple functional alterations

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and are involved in pathogenesis of atherosclerosis (11). During the development of atherosclerosis, ox-LDL stimulates transformation of macrophages and vascular smooth muscle cells into lipid-rich foam cells, induces proliferation and migration of vascular cells, and retards endothelial regeneration. At molecular level, ox-LDL is shown to promote expression of adhesion molecules, heat shock proteins, and coagulation proteins; to suppress production of endothelium-derived relaxing factor (nitric oxide) and prostacyclin; and to induce various proinflammatory cytokines and growth factors in vascular cells (12, 13).

Nuclear factor-κB (NF-κB) appears to play an important role in the transcriptional regulation of inflammatory proteins such as cyclooxygenase-2 (COX-2), intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), and E-selectin (14 – 17). NF-κB exists in the cytoplasm of unstimulated cells and bound to its inhibitory protein, IκB. Phosphorylation of IκB leads to its degradation and subsequent translocation of NF-κB to the nucleus where it activates transcriptions of target genes (18). In atherosclerosis process NF-κB functions as a director for pro-inflammatory and anti-inflammatory genes and as a regulator for cell survival and proliferation (14).

Although the *Ginkgo biloba* extracts possess some biologically protective effects against development of atherosclerosis, the mechanism remains to be further investigated. It is still unclear whether ginkgolide B can inhibit ox-LDL–induced inflammatory reactions in human umbilical vein endothelial cells (HUVECs). To address this issue, we investigated effects of ginkgolide B on expression of inflammatory proteins induced by ox-LDL in HUVECs and the intercellular signaling mechanisms.

Materials and Methods

Reagents

Anti-ICAM-1, IκB, phosphorylated-IκB, and β-actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Pyrrolidine dithiocarbamate, phenylmethylsulfonyl fluoride, and leupeptin were purchased from Sigma (St. Louis, MO, USA). Ginkgolide B was purchased from Daguanyuan Company (Daguanyuan Co., Xuzhou, Jiangsu, China).

Preparation of LDL and ox-LDL

Human LDL was isolated from fresh serum by sequential ultracentrifugation. LDL was oxidized with $CuSO_4$ (5 μM) for 16 h at 37°C, and then the oxidation was stopped by addition of EDTA (20 μM). The oxidation was confirmed by the thiobarbituric acid-reactive substance assay. The ox-LDL preparation was filtered through 0.22-μm filters and stored at 4°C. Protein concentration of ox-LDL was determined by spectrophotometer at the wavelength of 280 nm (UV-visible spectrophotometer; Shimadzu, Kyoto).

Cell culture

Human umbilical cords were obtained from healthy donors, from whom we received informed consents. HUVECs were isolated from fresh umbilical vein and cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, antibiomycins (10 μM penicillin G and 10 μM streptomycin) at 37°C in a humidified 5%-CO_2 atmosphere. HUVECs at passages 3 – 5 were used in the current study.

ox-LDL treatment

After being stimulated for various times with ox-LDL in serum-free medium, the cells were lysed by dissolving them in lysis buffer (1× = 1% Triton X-100, 100 mM Tris/HCl, pH 7.2, 50 mM NaCl, 5 mM EDTA, 2 mM vanadata, 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml of leupeptin), and then the samples were sonicated and centrifuged at 15,000 × *g* for 5 min. The lysates were subjected to immunoprecipitation or western blotting with specific antibodies.

Western blot analysis

Cell lysates were analysed with SDS-PAGE and electrotransfered to PVDF membranes. Membranes were then blocked with 1% bovine serum albumin for 1 h and incubated with specific antibodies for 2 h. After three washes in TPBS (containing 0.5% Tween 20 in PBS), the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies in PBST for 1 h. The bands were detected by chemiluminescence detection agents. Densitometry of the blot was performed and the bands were analyzed by Gene Genius Bio Imaging System (Gene Co., South San Francisco, CA, USA).

Immunofluorescence of NF-κB

NF-κB was detected by the NF-κB Activation, Translocation Assay Kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). Cells were seeded onto flame-sterilized coverslip placed in a 6-well tissue culture plate. After being treated with ox-LDL for 6 h, and the cells were fixed for 15 min in 4% (w/v) paraformaldehyde/PBS and made permeable by the addition of 0.2% Triton X-100/PBS for 15 min. Blocking solution was added at 4°C overnight and then anti-NF-κB 65 antibody was added in each well for 2 h. After washing 3 times, anti-rabbit IgG antibody conjugated

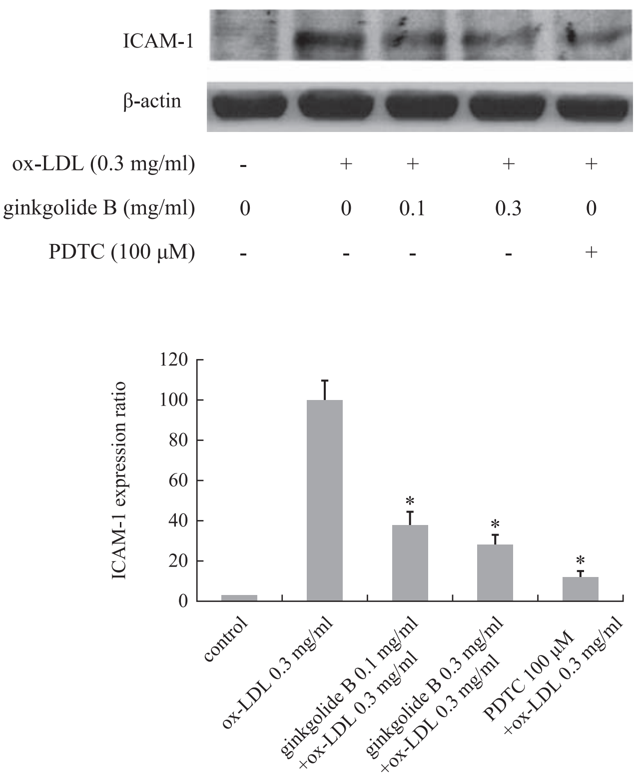

Fig. 1. Effects of ginkgolide B on ox-LDL–induced ICAM-1expression. Cells were preincubated with various concentrations of ginkgolide B for 1 h, and then treated with 0.3 mg/ml of ox-LDL for 6 h. The lysates were employed to determined abundance of ICAM-1 and *β*-actin by western blot analysis using specific antibodies as described in Materials and Methods. The data are representative of four independent experiments, and the intensities of the bands correspond to the levels of ICAM-1 and *β*-actin. **P*<0.05 indicates a significant difference between ginkgolide B–pretreated cells and the cells treated with ox-LDL alone.

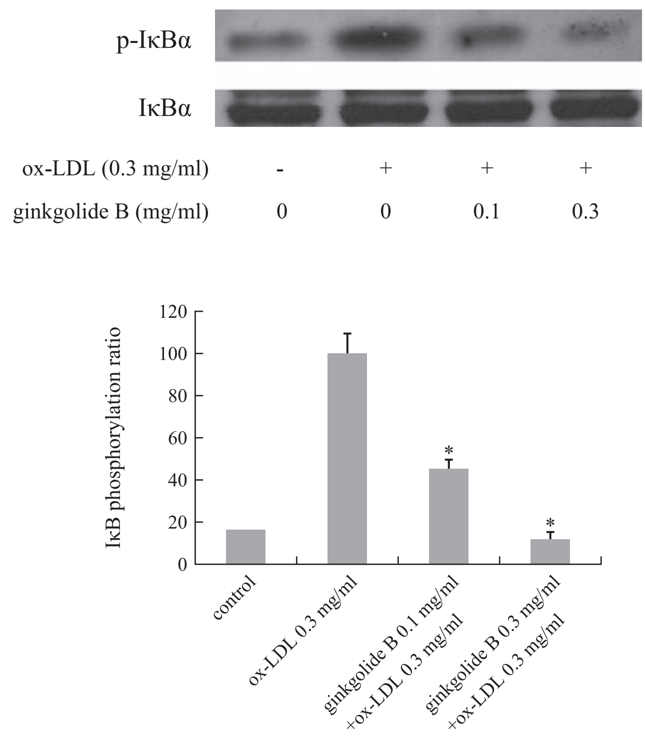

Fig. 2. Effects of ginkgolide B on ox-LDL–induced I*κ*B phosphorylation. Cells were preincubated with various concentrations of ginkgolide B for 1 h and then treated with 0.3 mg/ml of ox-LDL for 6 h. The lysates were used to determine phosphorylation of I*κ*B by Western blot analysis using specific antibodies. The data are representative of four independent experiments, and the intensities of the bands correspond to the levels of phosphorylated I*κ*B and I*κ*B. **P*<0.05 indicates a significance difference between ginkgolide B–pretreated cells and the cells treated with ox-LDL alone.

with Cy3 was added and incubated for 1.5 h. Cells were then incubated with DAPI for 20 min to stain the nuclei. NF-*κ*B p65 was imaged by a fluorescence microscope (BX60; Olympus, Ina). NF-*κ*B p65 was shown in red fluorescence and the nucleus, in blue fluorescence.

Preparation of nuclear extracts

Nuclear and cytoplasmic proteins were prepared using the Nuclear Cytoplasmic Extraction Reagents kit obtained from Beyotime Institute of Biotechnology. We followed the procedures described in the manufacturer's manual. Briefly, after washing with PBS, HUVECs were scraped off from cell culture dish with 0.2 ml of PBS and moved into the clear tube. After centrifugation at 500 × *g* for 5 min at 4°C, the cells were resuspended in 50 *μ*l of buffer A containing 1 mM PMSF, vortexed for 5 s, and then the lysates were placed on ice for 10 min. A 2.5-*μ*l aliquot of buffer B was added to the tube on ice for 1 min, and then the lysates were centrifuged at 12,000 × *g* for 5 min. After discarding the supernatant, pellets were re-suspended with 12.5 *μ*l buffer C containing 1 mM PMSF. The samples were placed on ice for 30 min and vortexed for 15 s every 2 min. The lysates were centrifuged at 16,000 × *g* for 10 min at 4°C. The supernatant fraction was collected and immediately transferred to a clean pre-chilled tube. The protein extracts were stored at –80°C until use.

Flow cytometry analysis of reactive oxygen species (ROS)

After incubating with 0.3 mg/ml of ginkgolide B for 1 h, the cells were stimulated with or without ox-LDL for 4 h. Cells were labeled with 10 *μ*M DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) for 30 min and then washed three times. The amount of ROS was determined as mean fluorescence intensity measured by flow cytometry (COULTER EPICS XL; Beckman Coulter, Inc., Fullerton, CA, USA).

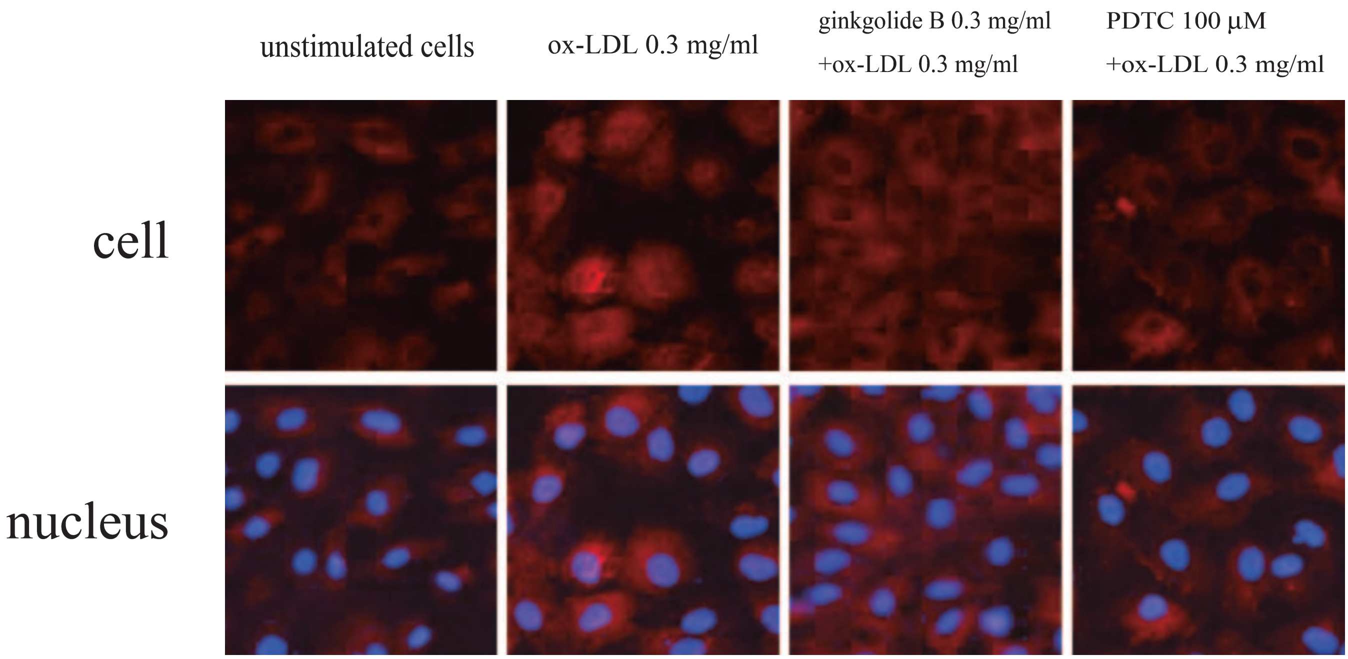

Fig. 3. Immunofluorescence analysis of NF-κB translocation induced by ox-LDL. Cells were grown on gelatin-coated six-well chamber glass slides. Cells were pretreated with 0.3 mg/ml of ginkgolide B or 100 μM PDTC for 1 h and then stimulated with 0.3 mg/ml of ox-LDL for 6 h. Subsequently, the cells were fixed and immunostained for NF-κB subunit (p65) and the nuclei as described in Materials and Methods. In the unstimulated cells, the NF-κB p65 subunit is predominantly localized in the cytoplasm, whereas cells stimulated with ox-LDL show significant translocation of p65 to the cell nucleus. In ox-LDL stimulated cells, pretreatment with ginkgolide B or PDTC, respectively, retains NF-κB p65 in the cytoplasm. Images are representatives of three independent experiments.

Statistical analyses

The data are from at least four experiments and are presented as the mean ± S.E.M. Statistical evaluation of the results was performed by the independent *t*-test and ANOVA with a Tukey post hoc test. The results were considered significant at a value of $P<0.05$.

Results

Ginkgolide B inhibited ICAM-1 expression induced by ox-LDL

It has been demonstrated that ox-LDL could stimulate endothelial cells to produce inflammatory proteins including ICAM-1. In our previous study we first detected that 0.1 and 0.3 mg/ml of ox-LDL could dose-dependently induce expressions of several proteins such as COX-2 (19). To be consistent with our previous finding, 0.3 mg/ml of ox-LDL was used in the study. As shown in Fig. 1, ox-LDL induced ICAM-1 expression, while ginkgolide B abolished ox-LDL–induced ICAM-1 expression in a dose-dependent manner. In addition, 100 μM pyrrolidine dithiocarbamate (PDTC), an inhibitory agent of NF-κB, also suppressed ICAM-1expression in HUVECs.

Ginkgolide B reduced IκB phosphorylation induced by ox-LDL

To examine whether the ginkgolide B regulates inflammatory gene expression induced by ox-LDL, IκB phosphorylation induced by ox-LDL was investigated because NF-κB signaling plays an important role in regulation of inflammatory gene expression. The level of IκB phosphorylation was increased by the stimulation of 0.3 mg/ml of ox-LDL. Ginkgolide B at concentrations of both 0.1 mg/ml and 0.3 mg/ml prevented IκB phosphorylation induced by ox-LDL in HUVECs (Fig. 2).

Ginkgolide B blocked NF-κB translocation

NF-κB is known to regulate inflammatory protein expression in multiple kinds of cells. Once it is activated, NF-κB translocates to the nucleus, and activates gene transcription. Since ginkgolide B reduced the level of IκB phosphorylation, we speculated that ginkgolide B might affect NF-κB activation. Therefore, intracellular movement of NF-κB was examined by immunofluorescence using NF-κB p65–specific antiserum as described in the Methods. As shown in Fig. 3, there was a significant nuclear translocation of NF-κB p65 in response to 0.3 mg/ml of ox-LDL stimulation. In

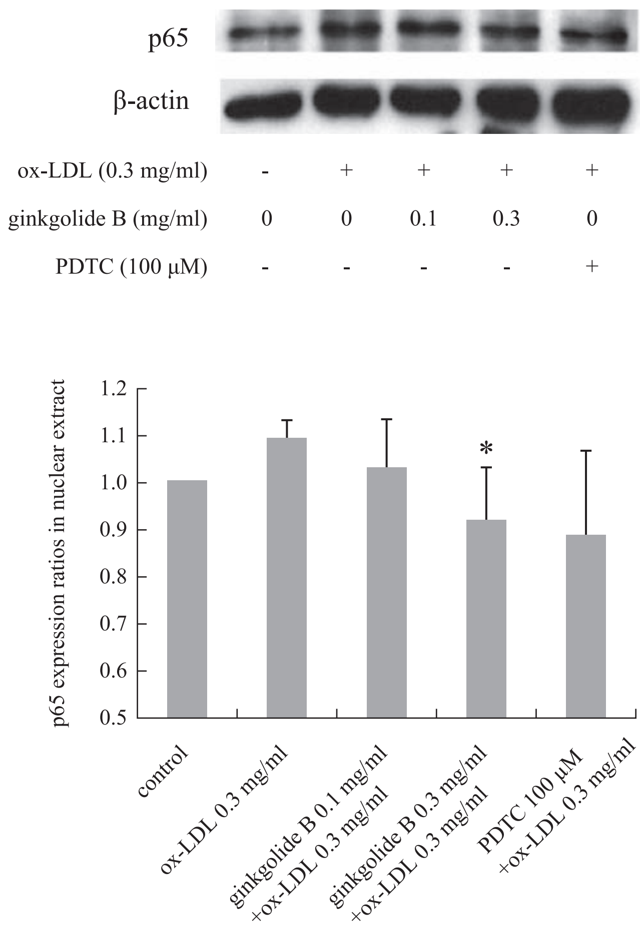

Fig. 4. Immunoblot analysis of NF-κB abundance in the nucleus induced by ox-LDL. Cells were pretreated with 0.1 or 0.3 mg/ml of ginkgolide B for 1 h and then stimulated with ox-LDL for 6 h. Nuclear extract was used to determine NF-κB abundance in the nucleus as described in Materials and Methods. The data are representative of three independent experiments. *P<0.05 indicates a significant difference between ginkgolide B–pretreated cells and the cells treated with ox-LDL alone.

contrast, NF-κB p65 was mostly retained in the cytoplasm of unstimulated cells. Moreover, ginkgolide B (0.3 mg/ml) significantly reduced NF-κB translocation to the nucleus. Similar results were obtained in 100 μM PDTC–treated cells. Moreover, we detected NF-κ expression in nuclear extract of HUVECs by immunoblot analysis. We found that ginkgolide B addition could effectively reduce NF-κB levels in the nuclear extract of HUVECs. Ginkgolide B at the concentration of 0.3 mg/ml almost completely prevented NF-κB translocation to the nucleus (Fig. 4).

Ginkgolide B decreased ROS induced by ox-LDL

Atherosclerosis is associated with increased intracellular oxidative stress. ox-LDL is believed to play a role in production of ROS in atherosclerosis. Therefore, we examined the inhibitory effects of ginkgolide B on ROS production. As shown in Fig. 5, exposure of HUVECs to ox-LDL (0.3 mg/ml) for 4 h significantly increased intracellular ROS production as measured by DCF fluorescence. In contrast, ox-LDL–induced ROS generation was significantly inhibited in cells pretreated with ginkgolide B at concentrations of 0.1 or 0.3 mg/ml for 1 h.

Discussion

Oxidative modified LDL is well-recognized to play a role in atherogenesis through several different signal transduction pathways (20). ox-LDL up-regulates endothelial MCP-1 via the lectin-like receptor pathway, which is involved in the activation of mitogen-activated protein kinase (MAPK), but not pertussis toxin-sensitive (PTX-sensitive) G proteins (21). However, it was also reported that biological activities of ox-LDL could also be mediated through a PTX-sensitive G protein–coupled receptor that involves activation of the Ras/Raf/MEK /MAPK pathway (22). In addition, previous studies indicated that ginkgolide B is an antagonist for PAF (6, 7). PAF is a lipid mediator and exerts multiple biological activities that are involved in thrombosis, vascular inflammation, and atherosclerosis. PAF receptor is also a G protein–linked heptaspanning receptor. It has been reported that ox-LDL contains PAF-like lipids, which are fragmented alkyl phosphatidylcholines. Therefore, it is suggested that ox-LDL and PAF could share their biological activities via a similar mechanism (23). Moreover, platelets play a central role in the thrombosis of atherosclerosis. There are multiple signal pathways for platelet activation induced by different platelet agonists. Although current antiplatelet drugs reduce cardiovascular events, clinicians still expect a more ideal agent for treatment and prevention of thrombosis. Recently Chintala et al. reported that antagonism of the proteinase-activated receptor 1 for thrombin may be a novel beneficial therapy for atherothrombotic disease (24). Ginkgolide B is an agonist of the PAF receptor, which can inhibit platelet aggregation induced by PAF. We also detected that ginkgolide B blocked platelet aggregation induced by collagen and adenosine diphosphate (ADP).

In the current study, we characterized the inflammatory response to ox-LDL in HUVECs and ginkgolide B suppression of the ox-LDL–induced inflammatory response and the signaling involved. ICAM-1 is a cell adhesion molecule and can recruit circulating leukocytes to vascular endothelial cells. Adhesion molecules expressed by endothelial cells modulate leukocyte–endothelium interactions, leading

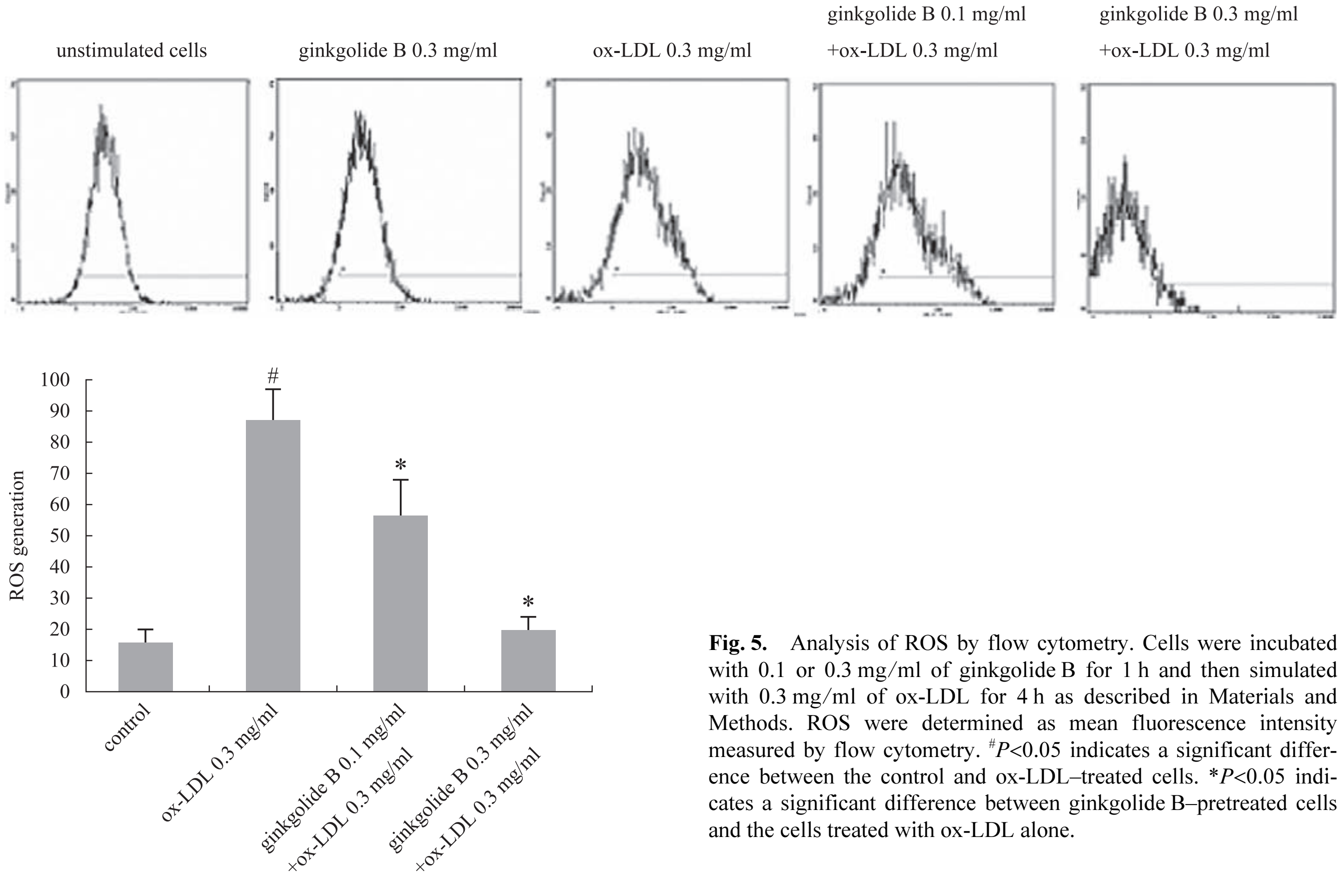

Fig. 5. Analysis of ROS by flow cytometry. Cells were incubated with 0.1 or 0.3 mg/ml of ginkgolide B for 1 h and then simulated with 0.3 mg/ml of ox-LDL for 4 h as described in Materials and Methods. ROS were determined as mean fluorescence intensity measured by flow cytometry. $^{\#}P<0.05$ indicates a significant difference between the control and ox-LDL–treated cells. $^{*}P<0.05$ indicates a significant difference between ginkgolide B–pretreated cells and the cells treated with ox-LDL alone.

to trans-endothelial migration of leukocytes, and stimulate proliferation of smooth muscle cells. Several evidences exist to support that ICAM-1 plays a crucial role in development of atherosclerosis plaque (25). In agreement with those studies, our results showed that ICAM-1 expression was significantly increased by ox-LDL stimuli. Ginkgolide B significantly attenuated ICAM-1 expression induced by ox-LDL. The results suggested that ginkgolide B reduces ox-LDL–induced inflammatory responses in HUVECs.

Since NF-κB is a regulator of inflammatory protein expression, it plays an important role in regulation of ICAM-1 expression. To determine whether ginkgolide B suppression of ICAM-1 expression is related to the NF-κB signaling pathway, we measured the levels of IκB phosphorylation, which is an upstream molecule in NF-κB activation. The results showed that IκB phosphorylation was enhanced by stimulation of ox-LDL, and ginkgolide B significantly reduced the ox-LDL–induced IκB phosphorylation. These findings implied that ginkgolide B might affect the NF-κB signaling pathway. Moreover, we investigated the effects of ginkgolide B on ox-LDL–induced NF-κB translocation to the nucleus. Several studies using the antibody specifically recognizing phosphorylated p65 showed that NF-κB activation occurred in smooth muscle cells, macrophages, and endothelial cells (26 – 28). Consistent with these findings, our results showed that ox-LDL–induced NF-κB migration to the nucleus and ginkgolide B could completely block NF-κB translocation. Similar results were also obtained by using PDTC. PDTC is an inhibitor of NF-κB, which regulates expression of several pro-inflammatory genes and some genes related to apoptosis. Furthermore, PDTC has been shown to be a potent antioxidant both in vitro and in vivo (29, 30). Cuzzocrea et al. showed that the inhibitory effect of PDTC on NF-κB activation was associated with inhibition of I-κB degradation (31). These findings imply that the effects of ginkgolide B on ox-LDL–induced ICAM-1 expression may be related to its blockage of associated NF-κB activation.

It has been demonstrated in several studies of cultured cells that ox-LDL is an important factor that enhances arterial cell apoptosis with involvement of both mitochondrial and death receptor pathways (Fas/Fas ligand, tumor necrosis factor receptors I and II) and the oxidative stress pathway (32 – 34). In the oxidative stress pathway, ox-LDL is able to induce ROS production in vascular smooth muscle cells (35). ROS have been documented to be involved in several major intracellular signaling pathways, which lead to changes in gene transcription and protein synthesis (36 – 38). Our results showed that ginkgolide B significantly inhibited ROS generation induced by ox-LDL, indicating that ginkgolide B possesses antioxidant activity.

In conclusion, the current study found that ginkgolide B attenuated the ox-LDL–induced expression of inflammatory protein – ICAM-1. Moreover, ginkgolide B also prevented IκB phosphorylation and NF-κB activation induced by ox-LDL in HUVECs. These findings suggested that ginkgolide B possesses some pharmacological effects on the inflammatory response induced by ox-LDL in HUVECs. The mechanisms of ginkgolide B action may be related to its inhibition of NF-κB activation and reduction of ROS production. Our present findings indicated some novel pharmacological activity of ginkgolide B, which might be used as a target in the prevention and treatment of atherosclerosis.

Acknowledgments

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