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ARTICLE

Sesamin Mitigates Inflammation and Oxidative Stress in Endothelial Cells Exposed to Oxidized Low-Density Lipoprotein

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Sesamin, a lignan from sesame oil, has been shown to have antihypertensive and antioxidative properties. This study examined the effects of sesamin on oxidized low-density lipoprotein (oxLDL)-induced endothelial dysfunction. Oxidative stress was determined by measuring the generation of intracellular reactive oxygen species (ROS) and by measuring the expression levels of superoxide dismutase (SOD) and endothelial nitric oxide synthase (eNOS). To assess the pro-inflammatory effects of oxLDL, ELISA was used to detect IL-8 expression, endothelin-1 (ET-1) secretion, and nuclear factor-κB (NF-κB) activation. The expression of adhesion molecules (ICAM-1, VCAM-1, and E-selectin) was examined by flow cytometry. In addition, several apoptotic signaling pathways were also investigated. The data showed that sesamin significantly ameliorated oxLDL-induced ROS generation and SOD-1 inactivation. Sesamin also attenuated the oxLDL-induced activation of NF-κB, suggesting that the inhibitory effects of sesamin on IL-8 and ET-1 release, adhesion molecule expression, and the adherence of THP-1 cells were at least partially through the blockade of NF-κB activation. Furthermore, sesamin attenuated oxLDL-induced apoptotic features, such as intracellular calcium accumulation and the subsequent collapse of mitochondrial membrane potential, release of cytochrome *c*, and activation of caspase-3. Results from this study may provide insight into possible molecular mechanisms underlying sesamin's beneficial effects against oxLDL-mediated vascular endothelial dysfunction.

KEYWORDS: Endothelium; oxLDL; sesamin; ROS; adhesion molecules; apoptosis

INTRODUCTION

Atherosclerosis is a chronic inflammatory process. The initiation of atherosclerotic lesion formation is caused by sublethal changes in endothelial function, called endothelial activation or dysfunction. Oxidative stress and reactive oxygen species (ROS) are believed to promote the conversion of low-density lipoprotein (LDL) to oxidized low-density lipoprotein (oxLDL), contributing to the pathogenesis of atherosclerosis. The early stages of the atherosclerotic process are initiated by accumulation of oxLDL and activation of endothelial cells with subsequent expression of adhesion molecules and increased binding of monocytes to the vascular endothelium. Proinflammatory cytokines, such as IL-8 and TNF-α, which are released when endothelial cells are exposed to oxLDL, up-regulate the expression of cell adhesion molecules (*1*). This series of adverse changes is also associated with a decrease in the bioavailability of nitric oxide (NO), a change that results in a reduced ability of the endothelium to control vessel tone. OxLDL further promotes vascular dysfunction by directly exerting cytotoxicity. Several lines of evidence have demonstrated that ROS play a central role in oxLDL-induced endothelial cell apoptosis (*2*). ROS such as superoxide ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) have been recognized as signaling molecules that stimulate cellular activities ranging from cytokine secretion to cell proliferation. At high concentrations, they can induce cell injury and death by oxidative modification of proteins, carbohydrates, nucleic acids, and lipids. In addition, the pro-apoptotic

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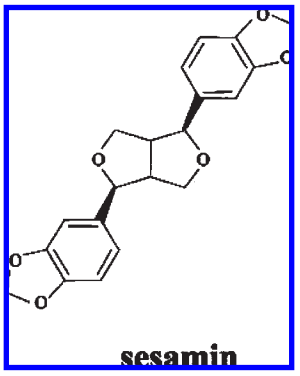

Figure 1. Chemical structure of sesamin.

effects of oxLDL-induced ROS in endothelial cells seem to involve the disturbance of mitochondrial membrane permeability followed by cytochrome *c* release, which finally activates the executioner caspases (*3*). In contrast, epidemiologic studies have demonstrated that dietary intake of antioxidants may reduce atherogenesis and improve vascular function by decreasing cellular production of ROS, thereby inhibiting endothelial dysfunction and maintaining the biologic activity of endothelium-derived NO (*4*).

Sesamin (**Figure 1**) is a major lignan constituent of sesame seeds and sesame oil. Several studies have shown that sesamin exerts antioxidative, anti-inflammatory, hepatoprotective, cholesterol-lowering, antihypertensive, anticancer, and neuroprotective effects (*5*−*9*). Recent studies have also demonstrated that sesamin metabolites induced vasorelaxation via an endothelial nitric oxide-dependent pathway (*10*), prevented deoxycorticosterone acetate (DOCA) salt-induced increases in NADPH oxidase activity (*11*), and inhibited atherosclerosis in LDL receptor-negative mice (*12*). Clinical studies on the effects of sesamin in humans have shown that sesamin reduces serum total and LDL cholesterol levels (*13*), suppresses the rise in plasma lipid peroxide level after high-intensity exercise (*14*), and reduces the adverse effects of smoking on the cardiac autonomic nervous system (*15*). Although sesamin has been reported to possess free radical scavenging activity and to inhibit oxidative modification of LDL and H_2O_2-induced ROS in red blood cells (*16*), to the best of our knowledge, no studies have been conducted on the effects of sesamin on oxLDL-induced endothelial dysfunction. We therefore investigated whether sesamin could protect against oxLDL-induced endothelial dysfunction via down-regulation of ROS-mediated signaling pathways.

In this study, we evaluated the effects of sesamin on ROS generation and the translation of NF-κB in cultured human umbilical vein endothelial cells (HUVECs) after exposure to oxLDL. In addition, we determined the levels of endothelial nitric oxide synthase (eNOS) expression, secretion of inflammatory cytokines, expression of adhesion molecules, and adherence of monocytic THP-1 cells to HUVECs. Furthermore, we also investigated the effects of sesamin on oxLDL-induced apoptosis of endothelial cells and the possible mechanisms involved in the signaling pathways, such as the accumulation of intracellular calcium, destabilization of mitochondria, and activation of caspase.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum, M199, and trypsin-EDTA were obtained from Gibco (Grand Island, NY); low serum growth supplement was obtained from Cascade Biologics (Portland, OR); 4,6-diamidino-2-phenylindole (DAPI), 2′,7′-bis(2-carboxyethyl-5 (and -6)-carboxyfluorescein-acetoxymethyl ester) (BCECF-AM), ethylenediaminetetraacetic

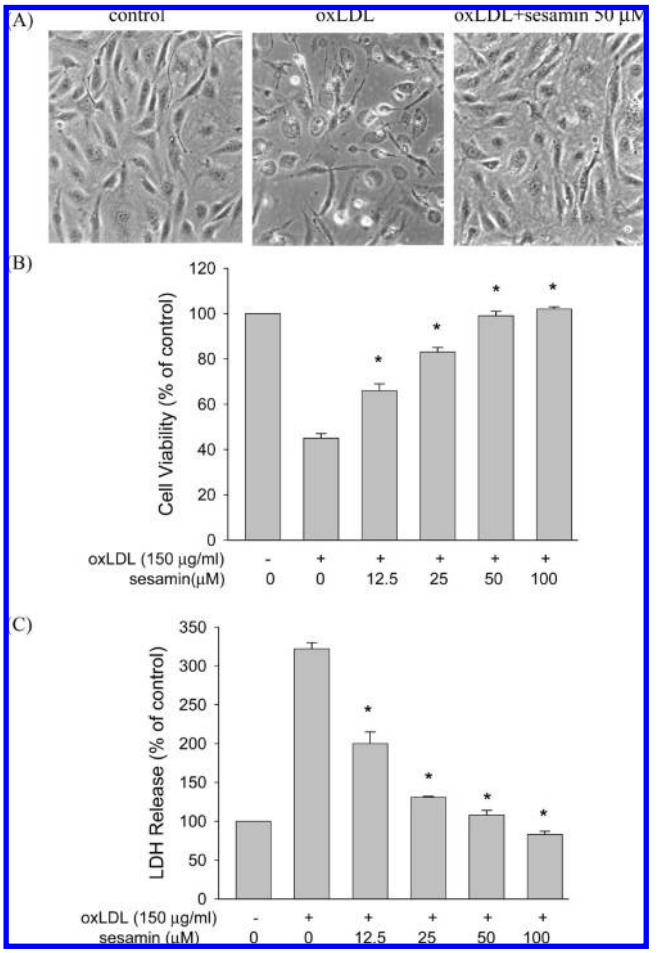

Figure 2. Effects of sesamin on oxLDL-induced endothelial cell death: (**A**) HUVECs were pretreated with indicated concentrations of sesamin for 2 h followed by stimulation with oxLDL (150 μg/mL) for another 24 h (photomicrographs are from phase-contrast microscopy); viability was determined via MTT assay (**B**) and lactate dehydrogenase (LDH) release (**C**). Data are expressed as the mean ± SEM of three independent analyses. ∗, $P < 0.05$ versus oxLDL treatment.

acid (EDTA), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], phenylmethanesulfonyl fluoride (PMSF), penicillin, and streptomycin were obtained from Sigma (St. Louis, MO); TUNEL (deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling) staining kit was obtained from Boehringer Mannheim (Mannheim, Germany); 2′,7′-dichlorofluorescein acetoxymethyl ester (DCF-AM), Fura-2 AM, and EnzChek caspase-3 assay kit were purchased from Molecular Probes (Eugene, OR); NF-κB/p65 ActivELISA kit was obtained from Imgenex Corp. (San Diego, CA); 5,58,6,68-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and antiactive caspase-3 were obtained from BioVision (Palo Alto, CA); antivascular cell adhesion molecule-1 (VCAM-1), anti-intercellular adhesion molecule-1 (ICAM-1), anti-E-selectin, and IL-8 ELISA kit were purchased from R&D Systems (Minneapolis, MN); anti-Cu/Zn superoxide dismutase (SOD-1) and anti-Mn superoxide dismutase (SOD-2) were obtained from Santa Cruz (Paso Robles, CA); and anti-eNOS, anti-Bcl 2, anti-Bax, anti-P53, and antiphosphorylated P53 were obtained from Transduction Laboratories (Lexington, KY). Sesamin (kindly provided by Jojia Bio-Tech Co. Inc., Kaohsiung, Taiwan) was purified from sesame oil. Briefly, 200 mL of sesame oil was dissolved in 1500 mL of acetone and stored at −70 °C overnight. The solidified triacylglycerol was discarded and the acetone solution collected after filtration. After removal of the acetone, the oil was saponified with 25 mL of ethanol containing 5% KOH for 1 h. The unsaponified fraction was diluted with distilled water and extracted three times with diethyl ether. A white crystalline powder containing 90% sesamin and 10% other lignans was obtained after another overnight diethyl ether

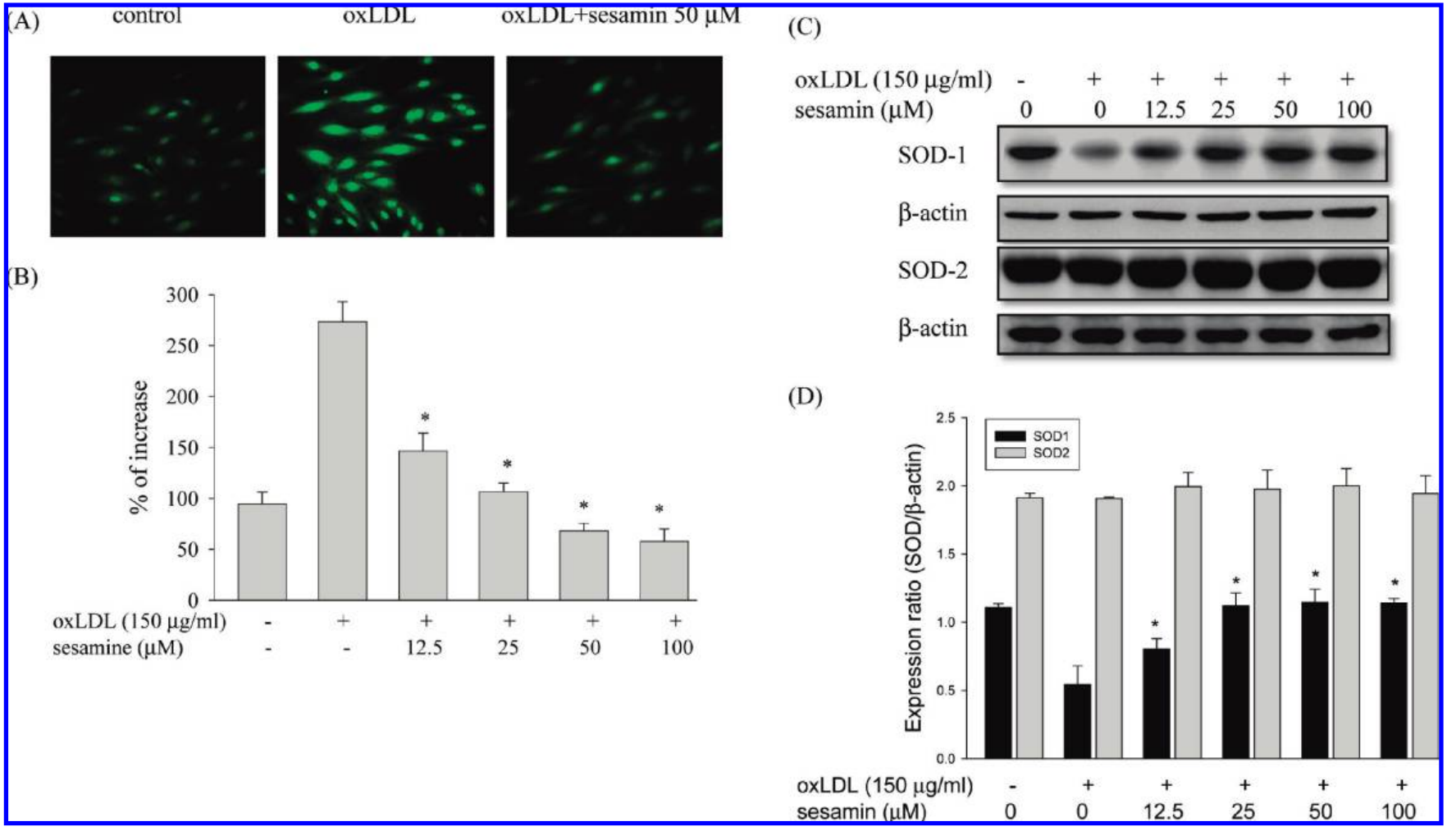

Figure 3. Inhibitory effects of sesamin on oxLDL-induced ROS production in HUVECs. After preincubation for 2 h with the indicated concentration of sesamin (12.5−100 μM), followed by a 1 h incubation with H_2O_2-sensitive fluorescent probe DCF-AM (10 μM), 150 μg/mL oxLDL were then added to medium for 2 h. (**A**) Fluorescence images show the ROS level in control cells (left) and HUVECs stimulated with oxLDL (middle) in the presence of 50 μM sesamin (right). (**B**) Fluorescence intensity of cells was measured with a fluorescence microplate reader. Fluorescence distribution of DCF-AM oxidation was expressed as a percentage of increased intensity. (**C**, **D**) Representative Western blots of Cu, Zn-SOD (SOD-1), and Mn-SOD (SOD-2) protein levels in HUVECs pretreated with sesamin for 2 h followed by stimulation with 150 μg/mL oxLDL for 24 h. Data are expressed as the mean ± SEM of three independent analyses. *, $P < 0.05$ compared with oxLDL-stimulated HUVECs.

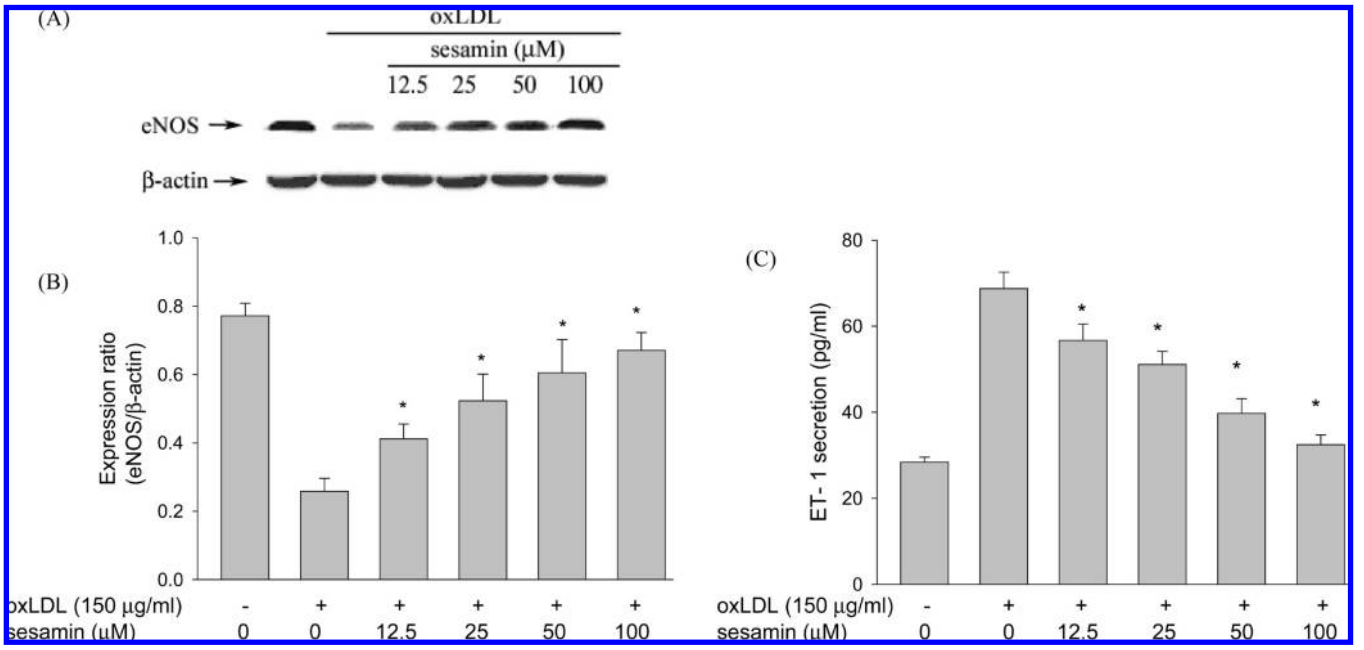

Figure 4. Western blot and ELISA measurement showing eNOS (A, B) and ET-1 (C) protein levels in HUVECs pretreated with indicated concentrations of sesamin followed by stimulation with oxLDL (150 μg/mL) for another 24 h. For Western blot analyses, a monoclonal anti-eNOS and a monoclonal anti-β-actin antibody (for normalization) were used. The values represent means $\pm$ SEM from three separate experiments. $*$, $P < 0.05$ versus oxLDL treatment.

extraction. The crude mixture was dissolved in chloroform and separated. Preparative thin-layer chromatography was carried out with chloroform/ethanol (9:1) to separate sesamin and other lignans (R_f 0.58). The purity of sesamin (exceeding 95%) was determined by high-performance liquid chromatography (HPLC). For the present experiments, sesamin was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mM as a stock solution.

Cell Cultures. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord with collagenase and used at passages 2−3 as described (*17*). Human umbilical cords were obtained from pregnant women with normal delivery who had no hepatitis, AIDS, or other contagious diseases. This study has been approved by a local ethical committee, and written informed consent was obtained from all of the subjects before collection of the umbilical cords. After dissociation, the cells were collected and cultured on gelatin-coated culture dishes in medium 199 with low serum growth supplement, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Subcultures were performed with trypsin−EDTA. Media were refreshed every second day. The identity of umbilical vein endothelial cells was confirmed by their cobblestone morphology and strong positive immunoreactivity to von Willebrand factor. THP-1, a human monocytic leukemia cell line, was obtained from ATCC (Rockville, MD) and cultured in RPMI with 10% FBS at a density of (2−5) $\times$ 10^6 cells/mL as suggested in the product specification sheet provided by the vendor.

Lipoprotein Separation and Oxidation. Native LDL was isolated and oxidatively modified from fresh normolipidemic human serum by sequential ultracentrifugations as previously described (*17*). Immediately before oxidation tests, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on a PD-10 Sephadex G-25 M gel (Pharmacia, St-Quentin, France) in phosphate-buffered saline (136.9 mM NaCl, 2.68 mM KCl, 4 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). The Cu^{2+}-modified LDL (1 mg protein/mL) was prepared by exposure of LDL to 10 μM $CuSO_4$ for 16 h at 37 °C. Protein levels were measured according to the method of Bradford (*18*).

Measurement of ROS Production. Our previous study showed that ROS generation is an upstream signal in oxLDL-mediated endothelial apoptosis (*17*). The effect of sesamin on ROS production in HUVECs was determined by a fluorometric assay using DCF-AM. Confluent HUVECs (10^4 cells/well) in 96-well plates were preincubated with various concentrations of sesamin for 2 h followed by loading with 10 μM DCF-AM for 1 h; the fluorescence intensity was measured with a fluorescence microplate reader (Labsystems) calibrated for excitation at 485 nm and emission at 538 nm (before and after 2 h of stimulation with 150 μg/mL oxLDL). The percentage increase in fluorescence per well was calculated by the formula $[(Ft_2 - Ft_0)/Ft_0] \times 100$, where Ft_2 is the fluorescence at 2 h of oxLDL exposure and Ft_0 is the fluorescence at 0 min of oxLDL exposure.

Imunnoblotting. To determine whether sesamin could ameliorate the oxLDL-induced apoptosis-regulating proteins, HUVECs were grown to confluence, pretreated with sesamin for 2 h, and then stimulated with oxLDL for 24 h. After treatment, cytosolic and mitochondrial protein fractions of cells were extracted as previously described (*17*). Protein was measured according to the Bradford method (*18*). Cytosolic SOD-1, SOD-2, eNOS, cytochrome *c*, and mitochondrial Bax and Bcl-2 expressions were determined by SDS-PAGE and immunoblot assay. The blots were developed using the enhanced chemiluminescence (ECL) kit (Amersham Life Science), followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h. To control equal loading of total protein in all lanes, blots were stained with mouse anti-β-actin antibody at a 1:50000 dilution. The bound immunoproteins were detected by an enhancer chemiluminescent assay (ECL; Amersham, Berkshire, U.K.). The intensities were quantified by densitometric analysis (Digital Protein DNA Imageware, Huntington Station, NY).

Adhesion Molecule Expression. To determine whether sesamin could modify oxLDL-induced adhesion molecule expression, HUVECs were grown to confluence and pretreated with sesamin for 2 h and then stimulated with oxLDL (150 μg/mL) for 24 h. At the end of stimulation, HUVECs were harvested and incubated with FITC-conjugated anti-VCAM-1, anti-ICAM-1, and anti-E-selectin for 45 min at room temperature. After HUVECs had been washed three times, their immunofluorescence intensity was analyzed by flow cytometry using a Becton Dickinson FACScan (Mountain View, CA).

Adhesion Assay. HUVECs at 1 $\times$ 10^5 cells/mL were cultured in 96-well flat-bottom plates (0.1 mL/well) for 1−2 days. Cells were then pretreated with the indicated concentrations of sesamin for 2 h followed by stimulation with oxLDL (150 μg/mL) for another 24 h. The medium was then removed, and 0.1 mL/well of THP-1 cells (prelabeled with 4 μM BCECF-AM for 30 min in RPMI at 1 $\times$ 10^6 cell/mL density) was added in RPMI. The cells were allowed to adhere at 37 °C for 1 h in a 5% CO_2 incubator. The nonadherent cells were removed by gentle aspiration. Plates were washed three times with M199. The number of adherent cells was estimated by microscopic examination, and then cells were lysed with 0.1 mL of 0.25% Triton X-100. The fluorescence intensity was

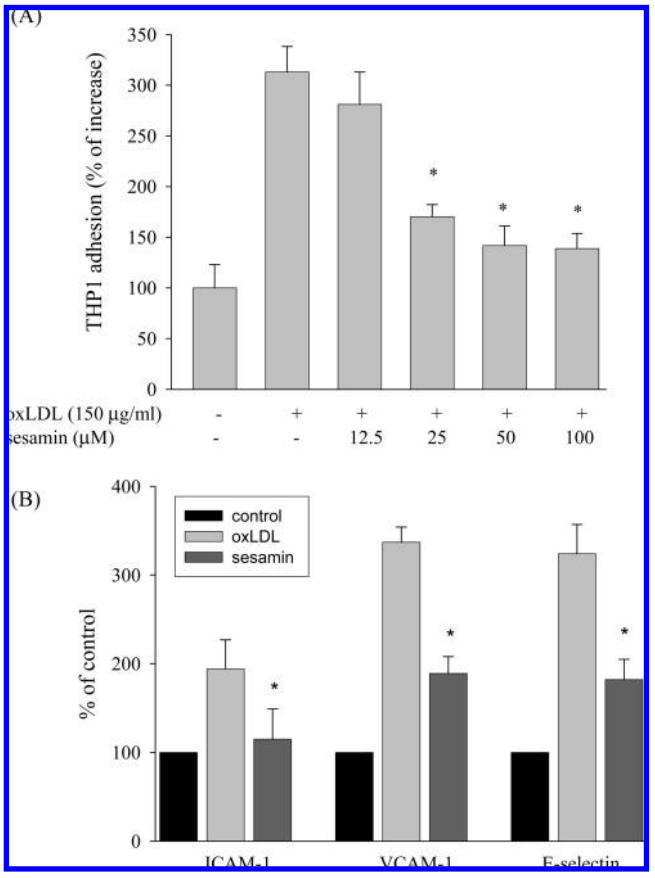

Figure 5. Effect of sesamin on oxLDL-induced adhesiveness of HUVECs to THP-1 monocytic cells and adhesion molecule expression. HUVECs were pretreated with indicated concentrations of sesamin for 2 h followed by stimulation with oxLDL (150 μg/mL) for another 24 h. (**A**) THP-1 cells preloaded with BECEF were incubated with HUVECs for 1 h. The adhesiveness of HUVECs to THP-1 was measured as described under Materials and Methods (**B**) Cell surface expression of ICAM-1, VCAM-1, and E-selectin was determined by flow cytometry. The values represent means ± SEM from three separate experiments. ∗, $P < 0.05$ versus oxLDL treatment.

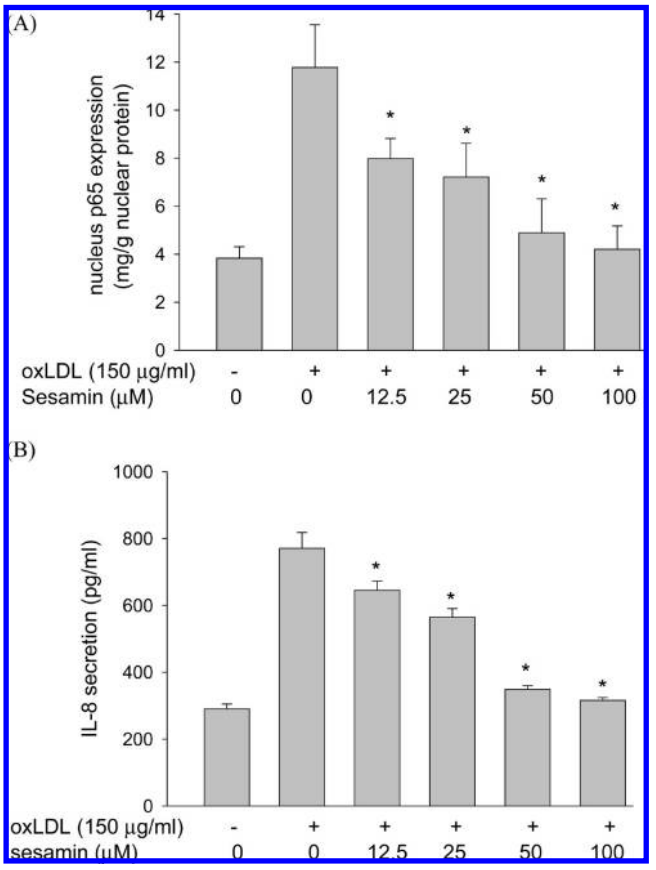

Figure 6. ELISA measurements showing NF-κB (**A**) and IL-8 (**B**) protein levels. HUVECs were pretreated with indicated concentrations of sesamin for 2 h followed by stimulation with oxLDL (150 μg/mL) for another 24 h. The values represent means ± SEM from three separate experiments. ∗, $P < 0.05$ versus oxLDL treatment.

measured at 485 nm excitation and 538 nm emission using a fluorescence microplate reader (Labsystems).

Assay for ET-1 and IL-8 Secretion. HUVECs were seeded in 24-well plates at 0.5×10^5 cells. After 2 days, cells were pretreated with the indicated concentrations of sesamin for 2 h followed by treatment with oxLDL (150 μg/mL) for 24 h. At the end of the oxLDL incubation period, cell supernatants were removed and assayed for ET-1 as well as IL-8 concentration using ELISA kits obtained from R&D Systems (Minneapolis, MN). Data were expressed in picograms per milliliter of duplicate samples.

NF-κB Assay. To prepare nuclear extracts for the NF-κB assay, the cells were first resuspended in buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM PMSF and then vigorously vortexed for 15 s and allowed to stand at 4 °C for 10 min. The samples were then centrifuged at 2000 rpm for 2 min. The pelleted nuclei were resuspended in 30 μL of buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF and incubated for 20 min on ice. The nuclear lysates were then centrifuged at 15000 rpm for 2 min. Supernatants containing the solubilized nuclear proteins were stored at −70 °C for subsequent NF-κB assay. The nuclear translocation of NF-κB was measured by an NF-κB p65 ActivELISA kit according to the manufacturer's instructions. The absorbance at 405 nm was determined using a microplate reader (SpectraMAX 340).

Determination of Apoptosis. To determine the effect of sesamin on the oxLDL-induced endothelial apoptosis, HUVECs were first incubated with sesamin (12.5−100 μM) for 2 h and then stimulated with oxLDL (150 μg/mL) for 24 h. At the end of stimulation, apoptotic cells were determined by TUNEL assay under fluorescence microscopy or flow cytometer.

Measurement of $[Ca^{2+}]_i$. To determine the effect of sesamin on the oxLDL-induced intracellular calcium rise, HUVECs were seeded onto 24 mm glass coverslips, pretreated with sesamin (12.5−100 μM) for 2 h, and then stimulated with oxLDL (150 μg/mL) for 24 h. The cells on coverslips were loaded with 2 μM Fura-2 AM (Molecular Probes) in M199 for 30 min at 37 °C. After loading, the cells were washed with HEPES buffer (in mM: NaCl, 131; KCl, 5; $CaCl_2$, 1.3; Mg_2SO_4, 1.3; KH_2PO_4, 0.4; HEPES, 20; glucose, 25; pH 7.4) to remove excess fluorescence dye. Then, the fluorescence of the cells from each coverslip was measured and recorded using an inverted Olympus microscope IX-70. $[Ca^{2+}]_i$ in endothelial cells was monitored by alternating excitation wavelengths between 340 and 380 nm and an emission wavelength of 510 nm with a Delta Scan System (Photon Technology International, Princeton, NJ) and calculated using Grynkiewicz's method (*19*).

Measurement of Mitochondrial Membrane Potential. To explore the effect of sesamin on the mitochondrial membrane potential ($\Delta\Psi_m$), the lipophilic cationic probe fluorochrome JC-1 was used. JC-1 exists either as a green fluorescent monomer at depolarized membrane potentials or as a red fluorescent J-aggregate at hyperpolarized membrane potentials. JC-1 exhibits potential-dependent accumulation in mitochondria, as indicated by the fluorescence emission shift from 530 to 590 nm. HUVECs were grown to confluence, pretreated with sesamin for 2 h, and then stimulated with oxLDL for 24 h; cells (5×10^4 cells/24-well plates) were rinsed with M199, and JC-1 (5 μM) was loaded. After 20 min of incubation at 37 °C, cells were examined under a fluorescent microscope. Determination of the $\Delta\Psi_m$ was also carried out using a FACScan flow cytometer.

Measurement of Active Caspase-3. To explore the effects of sesamin on oxLDL-induced caspase-3 activation, HUVECs were pretreated with

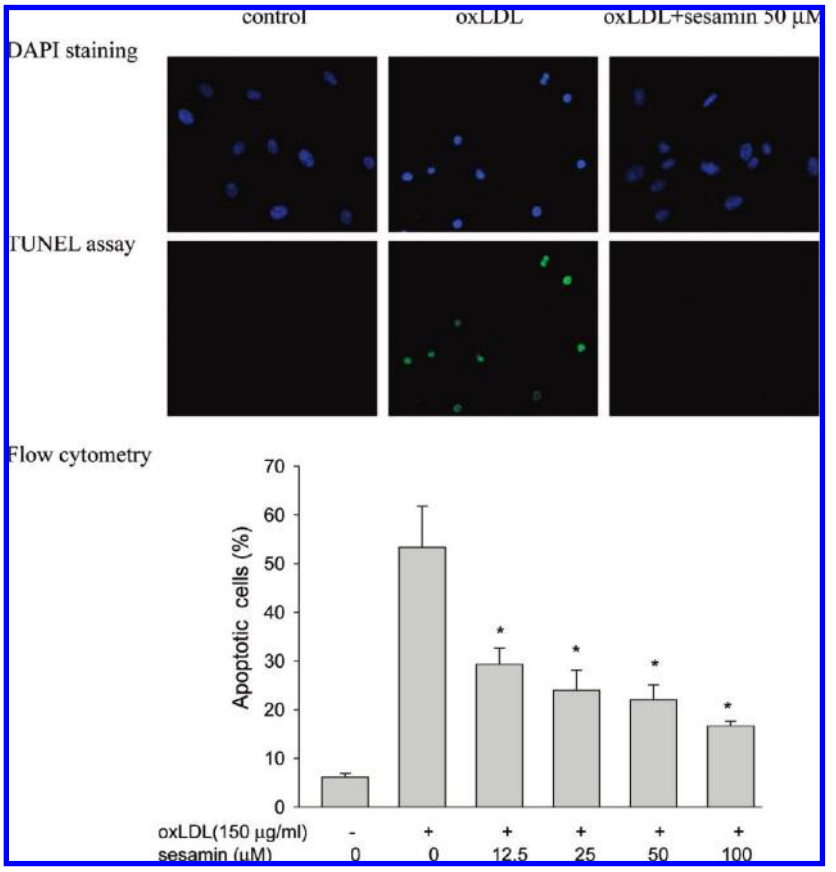

Figure 7. Effect of sesamin on oxLDL-induced endothelial cell apoptosis. HUVECs were pretreated without (middle) or with 50 μM sesamin (right) for 2 h followed by stimulation with oxLDL (150 μg/mL) for another 24 h: (top) cells stained with DAPI; (middle) cells stained using TUNEL assay. Fluorescence intensity of cells was measured with flow cytometry. Data are expressed as the mean ± SEM of three independent analyses. *, $P < 0.05$ versus oxLDL treatment.

sesamin for 2 h and then stimulated with oxLDL (150 μg/mL) for 24 h. The level of active caspase-3 was detected by flow cytometry using a commercial fluorescein active caspase kit (Mountain View, CA) under a fluorescence microscope. The activity of caspase-3 was also measured by an EnzChek caspase-3 assay kit according to the manufacturer's instructions (Molecular Probes Inc., Eugene, OR). After being lysed by repeated freeze−thaw cycles, cells were incubated on ice for 15 min and centrifuged at 15000*g* for 20 min. The protein concentrations of the supernatants were determined. Equal amounts of protein (50 μg) were added to the reaction buffer containing 5 mM caspase-3 substrate Z-DEVD-R110, and the mixture was incubated at room temperature for 30 min. The fluorescence generated from cleavage of the substrate by caspase-3 was monitored with a fluorescence microplate reader (Labsystems) calibrated for excitation at 496 nm and for emission at 520 nm.

Statistical Analyses. Results are expressed as mean ± SEM, and data were analyzed using one-way ANOVA followed by Student's *t* test for significant difference. A P value of < 0.05 was considered to be statistically significant.

RESULTS

Sesamin Inhibited OxLDL-Induced Cytotoxicity in HUVECs in a Concentration-Dependent Manner. The protective effect of sesamin on morphological features of cultured HUVECs after exposure to oxLDL was examined by phase-contrast microscopy. After a 24 h exposure to 150 μg/mL oxLDL, cells that had been co-incubated with sesamin demonstrated markedly less cytoplasmic shrinkage and membrane blebbing than cells that had not been exposed to sesamin (**Figure 2A**). The viability of cells incubated with oxLDL in the absence or presence of indicated concentrations of sesamin was assessed using the MTT assay, and membrane permeability was assayed by LDH release. The results of the assays revealed that the viability of HUVECs exposed to oxLDL was 55% less than that of control cells at 24 h and that the membrane permeability of HUVECs after 24 h of incubation with oxLDL was 322% greater than that of control cells; however, pretreatment with sesamin inhibited oxLDL-induced cytotoxicity of HUVECs dose dependently (all $P < 0.05$). The 50% effective concentration (ED_{50}) was calculated to be 21.1 μM for cell viability and 13.4 μM for cytotoxicity. Therefore, sesamin concentrations ranging from 12.5 to 100 μM were selected for the following experiments.

Sesamin Inhibited OxLDL-Induced ROS Generation in HUVECs. A previous study demonstrated that oxLDL evokes a progressive rise in cellular ROS, which subsequently leads to the activation of apoptotic signaling (*20*). We therefore investigated the effects of sesamin on the generation of ROS, a potential factor related to oxLDL-induced endothelial cell injury, by using DCF-AM as a fluorescence probe. Pretreatment of HUVECs with sesamin (12.5−100 μM) for 2 h before exposure to 150 μg/mL of oxLDL significantly decreased the level of ROS generation in a dose-dependent manner (all $P < 0.05$) (**Figure 3A**,**B**). ROS are able to inactivate antioxidative enzymes, leading to oxidative stress. We next turned our attention to the expression of SOD isoforms in endothelial cells in response to oxLDL. Our results showed that SOD-1, but not SOD-2, expression was diminished after treatment with oxLDL for 24 h; however, the expression level of SOD-1 increased in a dose-dependent manner after pretreatment with sesamin at concentrations ranging from 12.5 to 100 μM. (**Figure 3C**,**D**).

Sesamin Preserved eNOS Expression and Reduced ET-1 Secretion in Cells Exposed to OxLDL. Considerable evidence

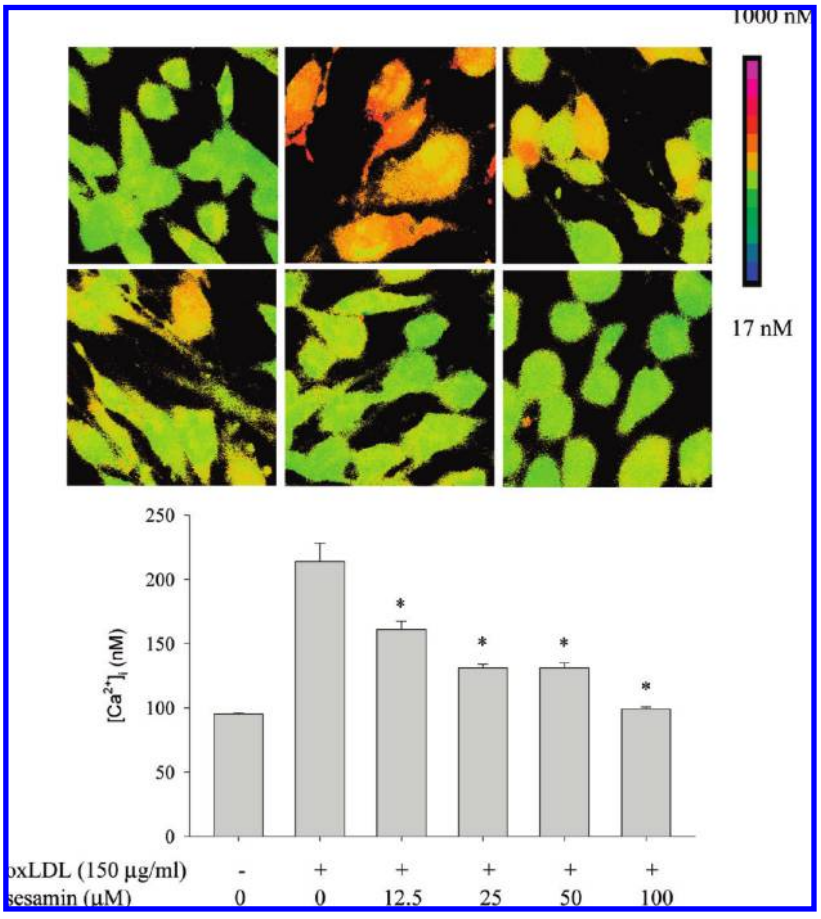

Figure 8. Effect of sesamin on cytoplasmic Ca^{2+} increase stimulated by oxLDL in Fura-2 AM-loaded HUVECs. Images were processed as indicated under Materials and Methods: (top left) control; (top middle) oxLDL; (top right) oxLDL + 12.5 μM sesamin; (bottom left) oxLDL + 25 μM sesamin; (bottom middle) oxLDL + 50 μM sesamin; (bottom right) oxLDL + 100 μM sesamin. Calcium changes are color coded (color bar) such that warm colors indicate high calcium. The values represent means ± SEM of more than 250 individual cells from three separate experiments. *, $P < 0.05$ versus oxLDL treatment.

indicates that oxLDL-induced endothelial dysfunction is associated with diminished expression of NO synthase (eNOS) and enhanced excretion of vascular contraction factor, ET-1. We therefore examined whether sesamin could influence the regulation of eNOS and ET-1 after treatment with oxLDL. As shown in **Figure 4A, B**, eNOS protein expression was significantly reduced in HUVECs after 24 h of incubation with oxLDL. Densitometric analysis revealed that the level of expression of eNOS in cells treated with 100 μM sesamin prior to oxLDL was almost 90% of the control level. The ET-1 concentration, however, was 2.4-fold higher in HUVECs that had been incubated for 24 h with oxLDL at 150 μg/mL (68.7 ± 3.8 pg/mL) than in control cells that had been incubated with the same concentration of native LDL (28.4 ± 1.1 pg/mL). Sesamin significantly inhibited the oxLDL-induced secretion of ET-1 secretion (all $P < 0.05$, **Figure 4C**).

Sesamin Suppressed OxLDL-Induced Adherence of THP-1 Cells to HUVECs and Expression of Adhesion Molecules. OxLDL damages endothelial cells by inducing the expression of adhesion molecules, which subsequently leads to the tethering, activation, and attachment of monocytes to endothelial cells (*21*). To test the effect of sesamin on monocyte adhesion to HUVECs, confluent monolayers of HUVECs were pretreated with various concentrations of sesamin for 2 h and then stimulated with oxLDL (150 μg/mL) for 24 h, followed by incubation with THP-1 cells for 1 h at 37 °C. As shown in **Figure 5A**, oxLDL stimulated the adherence of THP-1 cells to HUVECs; however, sesamin treatment inhibited this adhesion in a dose-dependent manner (12.5−100 μM). The effect of sesamin on the surface expression of adhesion molecules on HUVECs exposed to oxLDL was subsequently examined. As shown in **Figure 5B**, treatment with oxLDL (150 μg/mL) for 24 h significantly increased ICAM-1, VCAM-1, and E-selectin expression. Flow cytometry revealed that the induction of adhesion molecule expression was attenuated by 50 μM sesamin (all $P < 0.05$).

Sesamin Suppressed OxLDL-Induced Activation of NF-κB and Secretion of IL-8. The intracellular transduction pathway leading to expression of adhesion molecules following cytokine treatment involves activation of NF-κB (*22*). We evaluated whether the increase in the expression of adhesion molecules induced by oxLDL is associated with NF-κB translocation to the nucleus and whether sesamin interferes with the expected activation. As shown in **Figure 6A**, sesamin inhibited the oxLDL-induced NF-κB activation by nearly 100% at a concentration of 100 μM. The IL-8 concentration was 2.4-fold higher in HUVECs that had been incubated for 24 h with oxLDL at 150 μg/mL (771 ± 47 pg/mL) than in control cells that had been incubated with the same concentration of native LDL (316 ± 8 pg/mL), whereas sesamin significantly inhibited the oxLDL-induced secretion of IL-8 secretion (all $P < 0.05$, **Figure 6B**).

Sesamin Inhibited OxLDL-Induced Apoptosis of HUVECs. To further ascertain whether sesamin protects against oxLDL-induced endothelial apoptosis, we examined nuclear morphology with DAPI, a fluorescent DNA-binding agent, and in situ TUNEL assay for DNA fragmentation. As shown in **Figure 7**, cells incubated with oxLDL for 24 h showed typical features of apoptosis under fluorescence microscopy, including the forma-

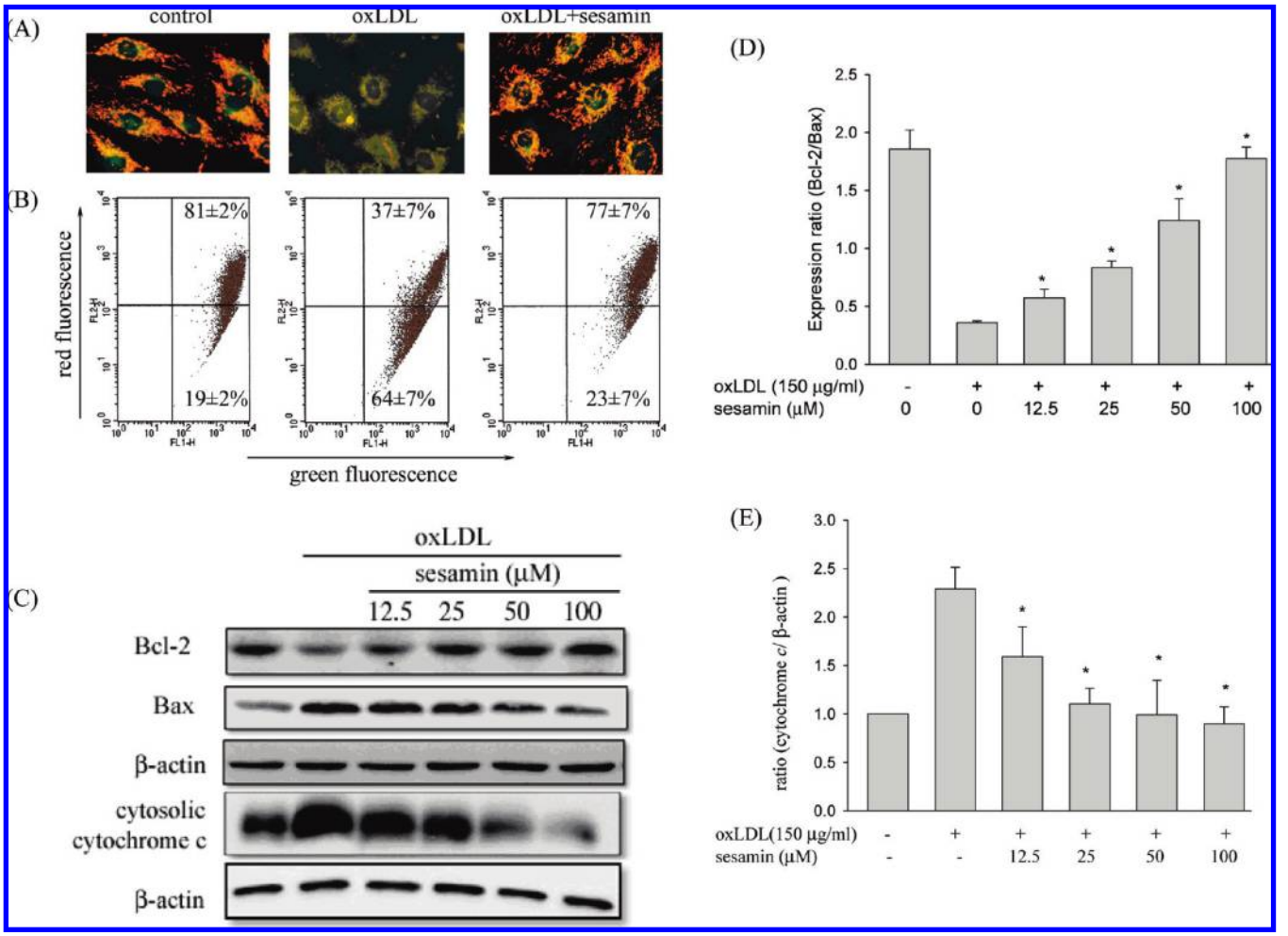

Figure 9. Effect of sesamin on mitochondrial transmembrane permeability transition. (**A**) $\Delta\Psi_m$ was assessed with the signal from monomeric and J-aggregate JC-1 fluorescence, as described under Materials and Methods: (left) control; (middle) oxLDL; (right) oxLDL + 50 μM sesamin. (**B**) JC-1 fluorescence was confirmed with flow cytometry: immunoblotting analysis of Bcl-2 protein family and mitochondrial cytochrome *c* release (**C**−**E**) in response to oxLDL and sesamin. HUVECs were pretreated with indicated concentrations of sesamin for 2 h followed by stimulation with oxLDL (150 μg/mL) for another 24 h. Representative Western blots and summary data show that oxLDL up-regulated pro-apoptotic (Bax) and down-regulated anti-apoptotic (Bcl-2) proteins and increased the concentration of cytochrome *c* in the cytosolic fraction. Pretreatment with sesamin suppressed these apoptosis-provoking alterations. Results were subjected to densitometric analysis; the values represent means ± SEM of three separate experiments. ∗, $P < 0.05$ versus oxLDL treatment.

tion of condensed and fragmented nuclei, which was not observed in the sesamin-pretreated HUVECs. This observation was confirmed by flow cytometry. Sesamin significantly inhibited oxLDL-induced DNA fragmentation in a dose-dependent manner (all $P < 0.05$).

Sesamin Reduced OxLDL-Induced Intracellular Calcium Accumulation. To understand the mechanisms underlying sesamin-inhibited oxLDL-induced apoptosis of HUVECs, we investigated the effect of chronic exposure of endothelial cells to oxLDL on intracellular calcium. We incubated HUVECs with 150 μg/mL of oxLDL in the absence or presence of sesamin. As shown in **Figure 8**, the basal $[Ca^{2+}]_i$ level increased from 95 ± 1 to 214 ± 13 nM in oxLDL-treated cells, and sesamin significantly inhibited the oxLDL-enhanced rise in intracellular calcium (all $P < 0.05$).

Effects of Sesamin on Mitochondrial Transmembrane Permeability Transition. To examine whether inhibition of mitochondrial disruption accounts for the anti-apoptotic effect of sesamin, we tested the effects of oxLDL on mitochondrial permeability. When HUVECs were exposed to oxLDL (150 μg/mL), the $\Delta\Psi_m$ was depolarized, as shown by the increase in green fluorescence (**Figure 9A**, middle panel). Pretreatment with sesamin reduced the change in $\Delta\Psi_m$, as indicated by the decrease in green fluorescence and restoration of red fluorescence (**Figure 9A**, right panel). The results from flow cytometry supported these findings. As seen in **Figure 9B**, oxLDL caused a marked increase in JC-1 green fluorescence (middle) compared to the control (left). Pretreatment with sesamin (50 μM) caused marked inhibition of this apoptotic index (right). Bcl-2 family proteins are upstream regulators of mitochondrial membrane potential. Because oxLDL depolarized the membrane potential and sesamin maintained it, we investigated whether sesamin also influenced the equilibrium of Bcl-2 family proteins. Immunoblotting studies demonstrated that oxLDL down-regulated the anti-apoptotic protein Bcl-2 and up-regulated the pro-apoptotic protein Bax. Sesamin pretreatment effectively repressed these oxLDL-evoked pro-apoptotic events (**Figure 9C**). Quantitative analysis showed that oxLDL significantly decreased the Bcl-2 to Bax ratio and that sesamin pretreatment preserved this anti-apoptotic index (**Figure 9D**).

It is known that disruption of mitochondrial membrane function results in the specific release of the mitochondrial enzyme cytochrome *c* into the cytosol. Therefore, cytosolic proteins were extracted and detected by Western blot. As shown in **Figure 9C**, the amount of cytochrome *c* released into the cytosolic fraction

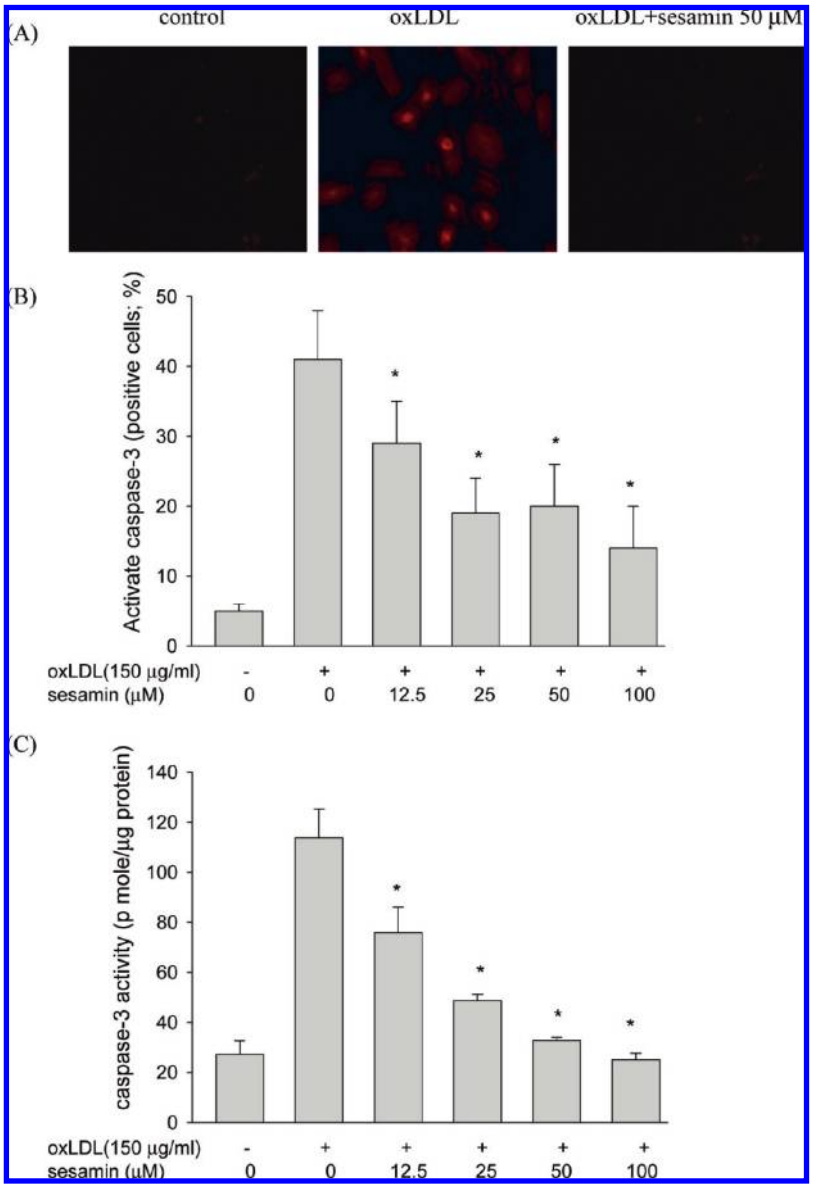

Figure 10. Effects of sesamin on oxLDL-induced caspase-3 activation. HUVECs were pretreated with indicated concentrations of sesamin for 2 h followed by stimulation with oxLDL (150 μg/mL) for another 24 h. (**A**). Fluorescent images show the activated caspase-3 level in control cells (left), in HUVECs stimulated with oxLDL (middle), and in the presence of 50 μM sesamin (right). (**B**) Fluorescence intensity of cells was measured with flow cytometry. (**C**) The activity of caspase-3 was 4.2-fold higher in oxLDL-treated HUVECs than in the control, but was limited to a 2.8-fold to 0.9-fold increase when pretreated with sesamin (12.5−100 μM). Data are expressed as the mean ± SEM of three independent analyses. ∗, $P < 0.05$ versus oxLDL treatment.

was much greater in HUVECs that had been incubated with oxLDL for 24 h than in control cells. The results indicate that sesamin significantly prevented oxLDL-induced release of cytochrome *c* (**Figure 9E**).

Sesamin Mitigated OxLDL-Mediated Caspase-3 Activation. Caspase-3 is a key factor in the execution of mitochondrial apoptosis (*23*). To examine whether oxLDL and sesamin ultimately influence this factor in modulating apoptosis, we determined the active form of caspase-3 by using fluorescence microscopy and flow cytometry. As shown in **Figure 10A**,**B**, active caspase-3 was significantly increased in cells that had been treated with oxLDL for 24 h. In contrast, the activation of caspase-3 by oxLDL was suppressed in cells that had been pretreated with 50 μM sesamin. The activity of caspase-3 was confirmed by using the EnzChek caspase-3 assay kit. The results showed that oxLDL led to a 4.1-fold increase in caspase-3 activity, whereas sesamin pretreatment effectively suppressed the activity of this apoptotic factor, implying a stimulatory effect of oxLDL and inhibitory action of sesamin on caspase-3 activity (**Figure 10C**, all $P < 0.05$).

DISCUSSION

OxLDL contributes to many atherogenic steps in the vessel wall. Considerable evidence indicates that oxLDL induces modification of cell protein structure, elicits ROS generation and peroxidation of cellular lipids, and alters the regulation of various signaling pathways and gene expression. In the present study, we demonstrated that sesamin ameliorated oxLDL-induced endothelial dysfunction by inhibiting inflammatory and oxidative damage that leads to cell apoptosis. Specifically, sesamin suppressed the generation of ROS, which subsequently attenuated the oxLDL-impaired expression of antioxidant enzymes, increased the bioavailability of NO, reduced ET-1 secretion, stabilized the mitochondrial membrane, and maintained the endothelial $[Ca^{2+}]_i$ level, thereby preventing the release of mitochondrial protein cytochrome *c*, a molecule required

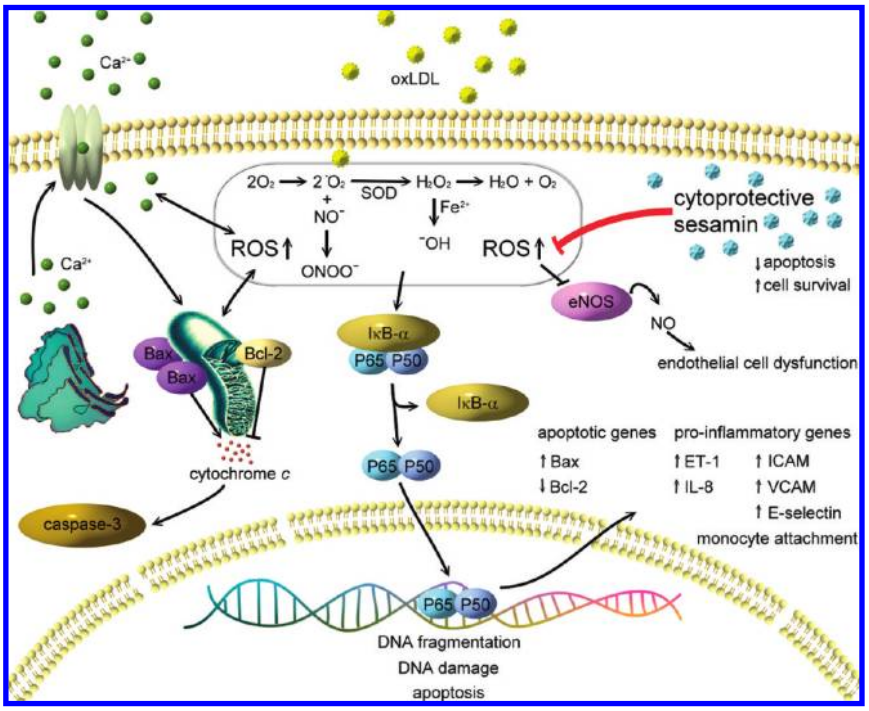

Figure 11. Schematic diagram showing cytoprotective signaling of sesamin in oxLDL-induced endothelial dysfunction. As depicted, sesamin inhibits the ROS-mediated signaling cascades induced by oxLDL. → indicates activation or induction, and —| indicates inhibition or blockade.

for the activation of the pro-apoptotic protein caspase-3 (**Figure 11**).

ROS have been shown to induce expression of multiple genes and to participate in the process of apoptosis, proliferation, and inflammation in almost all biological systems, including the cardiovascular system. Intracellular ROS levels are regulated by the balance between ROS-generating enzymes and antioxidant enzymes including superoxide dismutase, catalase, and glutathione peroxidase. In fact, many inflammatory mediators exert their actions through ROS accumulation. It has been shown that endothelial dysfunction caused by oxLDL is due to a decrease in antioxidative enzymes, thereby inducing apoptosis by activating multiple ROS-sensitive signaling pathways. Our findings that sesamin treatment significantly reduced ROS generation, which subsequently ameliorated oxLDL-attenuated SOD-1 expression, are consistent with results from a previously published study (*24*). Similar observations were reported by Daisuke et al. (*11*), who demonstrated that sesamin protected against vascular superoxide production in deoxycorticosterone (DOCA)-salt hypertensive rats. We assume that the main mechanism by which sesamin protects against oxLDL-induced endothelial dysfunction is its antioxidant action.

Endothelium plays a primary regulatory role by secreting substances that control both vascular tone and structure. One of these substances is NO. In addition to determining vessel tone, NO also acts as an antioxidant by reacting with superoxide anions to form peroxynitrite. In many vascular pathologies, a combination of altered rates of NO production along with an increased removal of NO leads to an apparent reduction in the bioavailability of NO. It has been shown that antithrombotic and antiatherosclerotic properties of NO are achieved by its ability to inhibit the expression of the cell surface adhesion molecules (*3*) and inhibit platelet adhesion under flow conditions (*25*). In normal physiology, superoxide is detoxified by the enzyme SOD, thereby preventing its interaction with NO. Results from our experiments showed that sesamin ameliorated the oxLDL-diminished expression of eNOS and had an inhibitory effect on the oxLDL-induced adhesiveness between monocytes and HUVECs. Findings from our study are also in agreement with an earlier investigation that reported that sesamin induced eNOS protein expression and increased the level of cGMP in endothelial cells (*26*). We further examined the inhibitory effects of sesamin on the oxLDL-induced surface expression of adhesion molecules in HUVECs. As expected, sesamin repressed the oxLDL-induced surface expressions of these adhesion molecules (ICAM, VCAM, and E-selectin). The findings of the present study are all in accordance with the antioxidative and anti-inflammatory effects of sesamin in response to oxLDL treatment in HUVECs.

NF-κB is often viewed as a global regulator of cytokines and mitogenic products that may influence vascular smooth muscle proliferation as well as endothelial apoptosis. Therefore, suppression of NF-κB can be expected to result in the prevention of atherogenesis. In accordance with a previous study that demonstrated that sesamin suppressed LPS-induced NF-κB activation and subsequent cytokine production, results from our observations indicate that suppression of NF-κB might be linked with anti-inflammation because subsequent secretion of IL-8 was significantly inhibited by pretreatment with sesamin (**Figure 6A**,**B**). Whether the effect of sesamin on suppression of oxLDL-induced NF-κB activation involves the activation of p38 mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K) requires further study.

It has been suggested that oxidative stress inhibits Ca^{2+}-ATPase. The inhibition of this transport protein results in a sustained elevation of $[Ca^{2+}]_i$, which leads to release of cytochrome *c* and subsequent loss of mitochondrial membrane potential (*27*). Free radical overproduction also increases the concentration of calcium in the cytosol, which contributes to the activation of endonucleases that degrade DNA (*27*). Accordingly, we believe that the anti-apoptotic effects of sesamin might result from its ability to inhibit the generation of ROS, which, in turn, would prevent the release of endothelial $[Ca^{2+}]_i$, thereby preventing the release of mitochondrial protein cytochrome *c*, a molecule required for the activation of the pro-apoptotic enzyme caspase-3 (*28*).

The concentrations of sesamin required to suppress oxLDL-induced endothelial dysfunction in our study were similar to those reported to inhibit other responses, such as proliferation in human tumor cells (*9*), the release of cytokins in lipopolysaccharide-activated microglia cells (*29*), and enodthelilin-1 production in HVUECs (*26*). It has been reported that metabolites of sesamin were detected in rat blood plasma at concentrations over 1 μM after feeding with a 1% sesamin diet for a month. The plasma concentration of the primary metabolite SC-1 (demethylpiperitol) was greater than 10 μM and was shown to induce vasorelaxation in rat aortic ring (*30*). The recommended daily dose of sesamin ingestion necessary to induce hypocholesterolemic effects is 60 mg (*13*). In humans, it is unclear how much the circulating blood level would be elevated by a single dose of sesamin because the pharmacokinetics of the metabolites of sesamin have not been completely established. It is also unknown whether prolonged use of sesamin would lead to chronic accumulation of some of its metabolites in different tissues.

In summary, the results from our experiments indicate that sesamin attenuates oxLDL-induced endothelial dysfunction primarily through its ability to suppress oxLDL-induced ROS generation and impairment of antioxidant enzymes. Sesamin inhibited the expression of IL-8 and the expression of adhesion molecules, at least partially through the blockade of NF-κB activation. Sesamin treatment also inhibited the adhesion of monocytes to HUVECs as well as inhibited oxLDL-induced apoptosis. It is likely that these beneficial effects contribute, at least in part, to the antiatherogenic action of sesamin.

ABBREVIATIONS USED

OxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; NF-κB, nuclear factor-κB; ICAM-1, intercellular adhesion molecule-1;VCAM-1, vascular cell adhesion molecule-1; LDL, low-density lipoprotein; NO, nitric oxide; DOCA, deoxycorticosterone acetate; HUVECs, human umbilical vein endothelial cells; DAPI, 4,6-diamidino-2-phenylindole; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMSF, phenylmethanesulfonyl fluoride; BCECF-AM, 2′,7′-bis-(2-carboxyethyl-5 (and -6)-carboxyfluorescein-acetoxymethyl ester); EDTA, ethylenediaminetetraacetic acid; TUNEL, deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling; DCF-AM, 2′,7′-dichlorofluorescein acetoxymethyl ester; JC-1, 5,58,6,68-tetraethylbenzimidazolcarbocyanine iodide; SOD-1, copper/zinc superoxide dismutase; SOD-2, manganese superoxide dismutase; HPLC, high-performance liquid chromatography; SC-1, demethylpiperitol.

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