行政院國家科學委員會專題研究計畫 成果報告

探討天然多酚類化合物及運動前處理對老鼠大腦缺氧/再灌氧傷害之保護作用

研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 97-2314-B-039-003-
執 行 期 間 ：97年08月01日至98年07月31日
執 行 單 位 ：中國醫藥大學物理治療學系

計畫主持人：歐秀中
共同主持人：王鐘賢

處 理 方 式 ：本計畫可公開查詢

中 華 民 國 98年10月22日

Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

BASIC RESEARCH STUDIES

Crude extracts of *Solanum lyratum* protect endothelial cells against oxidized low-density lipoprotein-induced injury by direct antioxidant action

Wei-Wen Kuo, PhD,[a] **Chih-Yang Huang, PhD,**[b,g] **Jing-Gung Chung, PhD,**[a,h] **Shun-Fa Yang, PhD,**[c] **Kun-Ling Tsai, MS,**[d] **Tsan-Hung Chiu, MD, PhD,**[e] **Shin-Da Lee, PhD,**[f,i] and **Hsiu-Chung Ou, PhD,**[f] *Taichung, Taiwan*

Background: **Oxidized low-density lipoprotein (oxLDL) is a proatherogenic molecule that accumulates in the vascular wall and contributes to the pathogenesis of vascular dysfunction early in the development of atherosclerosis. The whole plant of *Solanum lyratum* is a traditional Chinese medicine that has been used for centuries to treat cancer, tumors, and herpes. However, the cellular and molecular mechanisms of its antioxidant effects are still largely unknown. This study tested the hypothesis that *Solanum lyratum Thunberg* extract (SLE) could block oxLDL-induced endothelial dysfunction in cultured human umbilical vein endothelial cells (HUVECs). Possible mechanisms were explored.**

Methods: **Antioxidative activities of SLE were assayed by measuring the scavenging of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical and the inhibition of copper-mediated or cell-mediated LDL oxidation. Production of reactive oxygen species (ROS) and the expression of adhesion molecules were evaluated in HUVECs after exposure to oxLDL and treatment with SLE. Several apoptotic signaling pathways were investigated.**

Results: **SLE scavenged DPPH and also delayed the kinetics of LDL oxidation in a dose-dependent manner. SLE attenuated the level of oxLDL-induced ROS generation, diminished the expression of endothelial NO synthase (eNOS), and enhanced the expression of adhesion molecules (vascular cellular adhesion molecule-1, E-selectin, and monocyte chemotactic protein-1) and the adherence of monocytic THP-1 cells to HUVECs. OxLDL increased the concentration of intracellular calcium, disturbed the balance of the Bcl-2 protein family, destabilized the mitochondrial membrane potential, increased the amount of cytochrome *c* released into the cytosol, and increased the activation of caspase 3. These detrimental effects were ameliorated dose-dependently by SLE ($P < .05$).**

Conclusion: **Crude extracts of *Solanum lyratum* protect against oxLDL-induced injury in endothelial cells by direct antioxidant action. (J Vasc Surg 2009;50:849-60.)**

Clinical Relevance: **Atherosclerosis is a chronic inflammatory disease characterized by lipid-laden lesions within arterial blood vessel walls. Inhibiting the oxidation of low-density lipoprotein may be an effective way to prevent or delay the progression of atherosclerosis. This study underscores the potential clinical benefits and application of *Solanum lyratum* extract in controlling oxidized low-density lipoprotein-associated vascular injury and cardiovascular disease.**

Atherosclerosis is considered to be a chronic inflammatory disease. Oxidized low-density lipoprotein (oxLDL) is an important biomolecule that triggers and sustains atherosclerotic lesions. An initial event in atherosclerosis is the oxidation of LDL by free radicals, transition metals, and lipoxygenases, which involves cleavage of polyunsaturated fatty acids within the LDL particle. Oxidation of LDL can be achieved in vitro by incubation with copper ions or in a more physiologic manner by incubation with cultured endothelial cells.

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 endo-

From Department of Biotechnology,[a] Institute of Medical Science,[b] Graduate Institute of Clinical Medical Science,[d] Department of Obstetrics and Gynecology,[e] and Department of Physical Therapy and Graduate Institute of Rehabilitation Science,[f] China Medical University; and Institute of Medicine, Chung Shan Medical University,[c] Department of Health and Nutrition Biotechnology,[g] Department of Biotechnology,[h] Department of Healthcare Administration,[i] Asia University.

This study was supported by grants from the National Science Council (NSC 97-2314-B-039-003) and China Medical University (CMU-96-088, CMU-97-240), Taichung, Taiwan, Republic of China.

Competition of interest: none.

Reprint requests: Hsiu-Chung Ou, PhD, Department of Physical Therapy and Graduate Institute of Rehabilitation Science, China Medical University, Taichung, Taiwan No. 91, Shuch-Shih Road, Taichung 404, Taiwan (e-mail: ouhc@mail.cmu.edu.tw).

0741-5214/$36.00

Copyright © 2009 by the Society for Vascular Surgery.

doi:10.1016/j.jvs.2009.06.046

Author's personal copy

thelium. Monocytes recruited by endothelial cells invade the subintimal space by a complex mechanism characterized by the enhanced expression of chemoattractant interleukins, adhesion molecules, and various cell-membrane cytokine-receptors. Previous study demonstrated that oxLDL-induced reactive oxygen species (ROS) generation was an upstream signal.[1] A progressive rise of cellular ROS subsequently leads to an activation of apoptotic signaling,[2] which includes reduction of the mitochondrial transmembrane potential with concomitant release of the mitochondrial protein cytochrome *c* and subsequent activation of caspase-3.[3] The inhibition of LDL oxidation and oxLDL-induced ROS generation may prevent or delay the progression of atherosclerosis.

An Eastern crude drug, *Solanum lyratum,* has been used to treat cancers, tumors, and warts for centuries, and it corresponds to the European crude anticancer drug *Solanum dulcamara*.[4] The whole plant of *Solanum lyratum* has extensive clinical applications. The chemical constituents of *Solanum lyratum* were isolated and identified as aspidistrin, methylproto-aspidistrin, furostanol glycosides, and steroidal glycosides.[5-8]

Many biologic actions of the extract of *Solanum lyratum Thunberg* (SLE) have been previously reported. For example, SLE inhibits anaphylactic reaction[9] and modulates nitric oxide (NO) production by inhibiting protein kinase C activity.[10] SLE is also commonly used as an anticancer drug to treat cancers of the liver, lung, and esophagus.[11,12]

Studies on the anticancer mechanisms of SLE have shown that SLE induces HeLa cell apoptosis by up-regulating expression of Fas/FasL,[12] low expression of Bcl-2, and up-regulation of Bax,[13] promoting the formation of cyclic adenosine monophosphate (cAMP) and activating protein kinase A (PKA) in gastric cancer cells.[14] Kang et al[15] demonstrated that *Solanum lyratum Thunberg* exhibited hepatoprotective activity by preserving the glutathione content and the activity of superoxide dismutase (SOD) and by reducing the production of malondialdehyde (MDA), an end product of lipid peroxidation in hepatic tissue.

From this research, we hypothesized that SLE could protect against oxLDL-induced endothelial dysfunction by down-regulation of ROS generation. We undertook the current study to explore whether SLE could scavenge free radicals and prevent oxidative modification of LDL. We also evaluated the effects of SLE on oxLDL-induced ROS generation and determined other downstream events, including the levels of endothelial NO synthase (eNOS) expression, adherence of monocytic THP-1 cells to human umbilical vein endothelial cells (HUVECs), and adhesion molecules expression. Furthermore, several ROS-mediated apoptotic signalings, such as the accumulation of intracellular calcium and mitochondrial destabilization, and the activation of caspase-3 were also investigated.

METHODS

Plant material and preparation of crude extracts of *Solanum lyratum.* *Solanum lyratum* was cell grown and planted in the medicinal herb garden of China Medical University. The whole plant of *Solanum lyratum* (600 g) was extracted repeatedly with 50% ethanol at room temperature. The combined ethanol extracts were filtered and evaporated under reduced pressure to yield a brownish viscous residue (58.44 g). For the present experiments, the crude extracts were dissolved in dimethyl sulfoxide (DMSO).

Cell cultures. HUVECs were isolated with collagenase and used at passage 2 to 3 as previously described.[16] After dissociation, the cells were collected and cultured on gelatin-coated culture dishes in medium 199 with low serum growth supplement, penicillin (100 IU/mL), and streptomycin (0.1 mg/mL). Subcultures were performed with trypsin-ethylenediaminetetraacetic acid (EDTA). Media were refreshed every 2 days. THP-1, a human monocytic leukemia cell line, was obtained from ATCC (Rockville, Md) and cultured in Roswell Park Memorial Institute (RPMI) media with 10% fetal bovine serum at a density of 2 to 5 $\times$ 10^6 cells/mL, as suggested by the vendor's product specification sheet.

Free radical scavenging activity of 1,1-diphenyl-2-picryl-hydrazyl. The radical scavenging capacity of SLE was determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) scavenging method. This spectrophotometric assay uses the stable radical DPPH• as a reagent.[17] The capability to scavenge the DPPH• radical was calculated using the following equation: DPPH• scavenging effect (%) = $[1 - (AS/AC)] \times 100$, where *AC* is the absorbance of the control (0.5 mL of 0.1mM ethanol solution of DPPH without SLE), and *AS* is the absorbance in the presence of SLE.

Lipoprotein and oxLDL preparation. Native LDL was isolated and oxidatively modified from fresh normolipidemic human serum by sequential ultracentrifugations, as previously described.[16] The EDTA-free LDL was immediately used for the measurement of the kinetics of LDL oxidation by continuously monitoring the change in the absorbency of conjugated diene at 232 nm at 37°C for up to 6 hours. In some experiments, LDL oxidation was estimated by measuring the production of thiobarbituric acid reactive substances (TBARS), as described previously.[18] MDA, an end product of lipid peroxidation, was used as a standard.

Measurement of LDL oxidation in HUVECs. Once HUVECs had reached confluence in 24-well plates, culture medium was removed and the cells were washed twice with M199 medium. The cells were then incubated with native LDL (100 μg/mL) in phenol red free medium in the presence of various concentrations of SLE for 24 hours. At the end of the incubation, media were collected and the levels of TBARS were measured as an index of LDL oxidation.

Measurement of ROS production. ROS have been implicated in the pathophysiology of many vascular disorders. The effect of SLE on ROS production in HUVECs was determined by a fluorometric assay using 2,′ 7′-dichlorofluorescein acetoxymethyl (DCF-AM) ester (Molecular Probes, Eugene, Ore). Confluent HUVECs (10^4

Author's personal copy

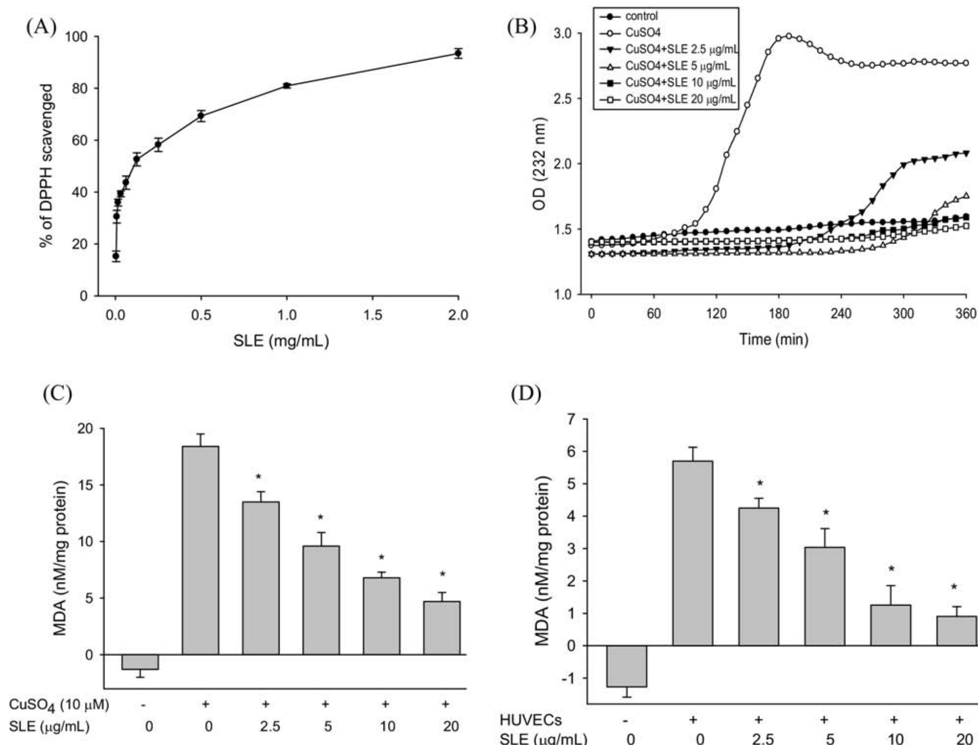

Fig 1. Antioxidative activities of *Solanum lyratum* extract *(SLE)*. The 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity of SLE was determined by incubating various concentrations of SLE with 1 mM DPPH in a total volume of 1 mL ethanol for 30 minutes. **A,** Change in the absorbance at 517 nm was recorded. The effect of SLE on Cu^{2+}-induced oxidation of low-density lipoprotein was determined by measuring **(B)** the prolongation of lag time of diene formation and **(C)** level of reduction in thiobarbituric acid reactive substances (TBARS) concentration after incubation for 16 hours. **D,** The effect of SLE on reduction in TBARS concentration of low-density lipoprotein after incubation with human umbilical vein endothelial cells for 24 hours. Data are shown as the mean ± standard error of three independent analyses. *$P < .05$ compared with control.

cells/well) in 96-well plates were preincubated with various concentrations of SLE for 2 hours. OxLDL was then added to the media in the absence or presence of SLE for 2 hours. After the removal of media from wells, cells were incubated with 10μM DCF-AM for 1 hour.

The fluorescence intensity was measured with a fluorescence microplate reader (Labsystem, Ramsey, Minn) calibrated for excitation at 485 nm and emission at 538 nm. The percentage of 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 hours of oxLDL exposure and Ft_0 is the fluorescence at 0 minutes of oxLDL exposure.

Immunoblotting. To determine whether SLE could ameliorate the oxLDL-induced apoptosis-regulating proteins, HUVECs were grown to confluence, pretreated with SLE for 2 hours, and then stimulated with oxLDL for 24 hours. After treatment, cytosolic and mitochondrial protein fractions of cells were extracted as previously described.[16] Protein was measured by the Bradford method.[19] Cytosolic SOD-1, SOD-2, eNOS, and cytochrome *c,* as well as mitochondrial Bax and Bcl-2 expressions, were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot assay. The blots were incubated with primary antibodies (1:1000) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour. To control equal loading of total protein in all lanes, blots were stained with mouse anti-β-actin antibody at a dilution of 1:50000. The bound immunoproteins were detected by an ECL kit (Amersham, Berkshire, UK). The intensities were quantified by densitometric analysis (Digital Protein DNA Imagineware, Huntington Station, NY).

Adhesion assay. Vascular inflammation is caused by the increase in leukocyte-endothelium adhesion by an upregulation of endothelial cell adhesion molecules. To determine whether SLE could reduce the oxLDL-induced adherence of monocytic cells and endothelial cells, HUVECs (1×10^5 cells/mL) were cultured in 96-well flat-bottom plates (0.1 mL/well) for 1 to 2 days. Cells were pretreated with 10 μg/mL of SLE for 2 hours, followed by oxLDL (130 μg/mL) for 24 hours. The media was then removed, and 0.1 mL/well of THP-1 cells (prelabeled with BCECF-AM 4 μM for 30 minutes in RPMI at 1×10^6 cells/mL density) were added in RPMI. The cells were allowed to adhere at 37°C for 1 hour in a 5% carbon

Author's personal copy

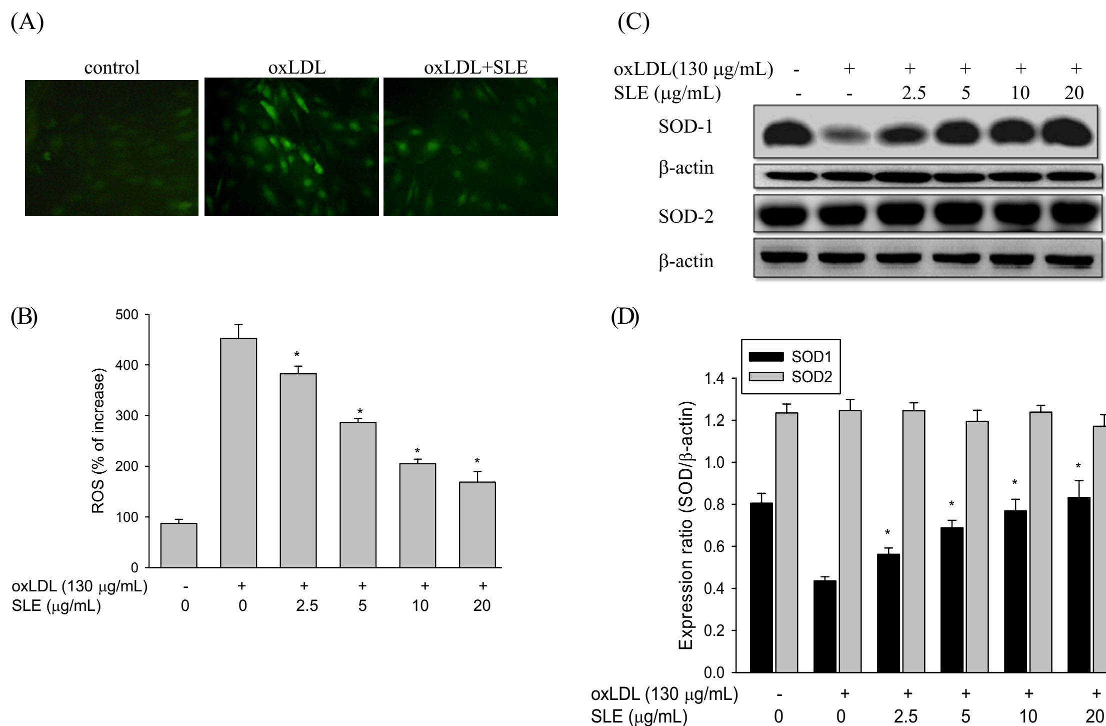

Fig 2. Inhibitory effects of *Solanum lyratum* extract *(SLE)* on oxidized low-density lipoprotein (*oxLDL*)-induced reactive oxygen species (ROS) production in human umbilical vein endothelial cells (HUVECs). After preincubation for 2 hours with the indicated concentration of SLE (2.5 to 20 μg/mL), oxLDL (130 μg/mL) was added to medium for 2 hours, followed by a 1-hour incubation with H_2O_2-sensitive fluorescent probe dichlorofluorescein acetoxymethyl ester (DCF-AM; 10μM). **A,** Fluorescence images show the ROS level in control cells *(left)* and HUVECs stimulated with oxLDL *(middle)* in the presence of 10 μg/mL SLE *(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 and D,** Representative Western blots of Cu, Zn-superoxide dismutase (*SOD*; SOD-1) and Mn-SOD (SOD-2) protein levels in HUVECs pretreated with SLE for 2 hours, followed by oxLDL (130 μg/mL) for 24 hours. Data are expressed as the mean ± standard error of three independent analyses. *$P < .05$ compared with oxLDL-stimulated HUVECs.

dioxide 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 lysed with 0.1 mL 0.25% Triton X-100. The fluorescence intensity was measured with a fluorescence microplate reader (Labsystem) calibrated for an excitation at 485 nm and for emission at 538 nm.

Adhesion molecule expression. To determine whether SLE could attenuate the level of adhesion molecule expression induced by oxLDL, HUVECs were grown to confluence, pretreated with SLE (10 μg/mL) for 2 hours, and stimulated with oxLDL (130 μg/mL) for 24 hours. The HUVECs were harvested and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-vascular cell adhesion molecule-1 (VCAM-1), anti-E-selectin, and anti-monocyte chemotactic protein-1 (MCP-1; R&D, Minneapolis, Minn) for 45 minutes at room temperature. After the HUVECs had been washed three times, their immunofluorescence intensity was analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif).

Measurement of $[Ca^{2+}]_i$. Intracellular calcium rise is involved in oxLDL-induced endothelial apoptosis.[20] Therefore, the effects of SLE on the increase in oxLDL-induced intracellular calcium concentration $[Ca^{2+}]i$ were determined. HUVECs were seeded onto 24-mm glass coverslips, pretreated with SLE for 2 hours, and then stimulated with oxLDL (130 μg/mL) for the indicated time periods. The cells on the coverslips were loaded with 2 μM fura-2 AM (Molecular Probe) in M199 and allowed to stand for 30 minutes at 37°C. The fluorescence of the cells on each coverslip was measured and recorded using an inverted Olympus IX-70 microscope (Olympus, Tokyo, Japan). The concentration of $[Ca^{2+}]i$ in endothelial cells was monitored with a Delta Scan System (Photon Technology International, Princeton, NJ) at an emission wavelength of 510 nm, with excitation wavelengths alternating between 340 and 380 nm, and calculated using Grynkiewicz's method.[21]

Author's personal copy

Measurement of mitochondrial membrane potential. The lipophilic cationic probe fluorochrome 5,58,6,68-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to explore the effects of SLE on mitochondria membrane potential ($^{\Delta}\Psi$m). JC-1 exhibits potential-dependent accumulation in mitochondria, as indicated by the fluorescence emission shift from 530 to 590 nm. After cells were treated with oxLDL (130 μg/mL) for 24 hours in the presence or absence of various concentrations of SLE, cells (5×10^4 cells/24-well plates) were rinsed with M199, and JC-1 (5 μM) was loaded. Cells were incubated 20 minutes at 37°C and examined under a fluorescence microscope. Determination of the $^{\Delta}\Psi$m was done using a FACScan flow cytometer.[22]

Measurement of active caspase-3. To explore the effects of SLE on oxLDL-induced caspase-3 activation, HUVECs were pretreated with SLE for 2 hours and then stimulated with oxLDL (130 μg/mL) for 24 hours. The level of active caspase-3 was detected by flow cytometry using a commercial fluorescein active caspase kit (BioVision, Mountain View, Calif) 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). The fluorescence generated from cleavage of the substrate by caspase-3 was monitored with a fluorescence microplate reader (Labsystem) calibrated for excitation at 496 nm and for emission at 520 nm.

Determination of apoptosis. To determine the effect of SLE on oxLDL-induced cytotoxicity, HUVECs were first incubated with SLE for 2 hours and then stimulated with oxidized LDL for 24 hours. Apoptotic cells were assessed by a terminal deoxynucleotide transferase-mediated deoxy uridine triphosphate nick-end labeling (TUNEL) assay under a fluorescence microscope or in a flow cytometer.

Statistical analyses. All experiments were repeated three or more times, and one of these results is provided. Results are expressed as mean ± standard error. Differences between the groups were analyzed using one-way analysis of variance, followed by t test. A value of $P < .05$ was considered statistically significant.

RESULTS

DPPH radical scavenging and oxidation of LDL. An SLE concentration of 0.11 mg/mL was required for scavenging 50% of the DPPH radicals (Fig 1, *A*). The copper-induced oxidation of LDL in the presence of various concentrations of SLE was determined by following the kinetics of conjugated diene formation and MDA formation. In the presence of various concentrations of SLE (2.5 to 20 μg/mL), the sigmoidal curves of diene formation shifted to the right, indicating that the oxidation of LDL was delayed (Fig 1, *B*). The effective concentrations of SLE that inhibit copper-induced LDL oxidation were subsequently studied by measuring the concentrations of TBARS. Incubation of LDL with copper for 16 hours resulted in significantly increased levels of MDA (−1.3 ± 0.7 nM/mg for native LDL and 18.4 ± 1.1 nM/mg for copper-treated LDL; Fig 1, *C*). MDA levels in the SLE-treated groups were decreased in a dose-dependent manner. SLE at the dose of 20 μg/mL significantly reduced the MDA level to 4.7 ± 0.8 nM/mg protein (Fig 1, *C*). As expected, the inhibitory effect of SLE on endothelial cell-mediated LDL oxidation was very similar (Fig 1, *D*). These results indicate that SLE is a potent antioxidant.

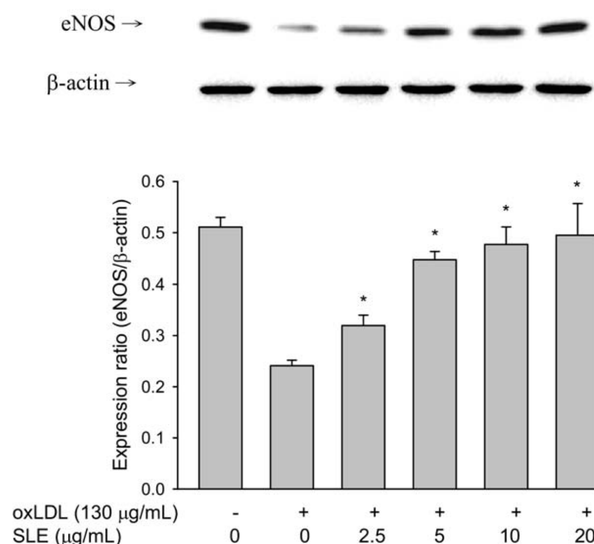

Fig 3. Effects of *Solanum lyratum* extract *(SLE)* on oxidized low-density lipoprotein *(oxLDL)*-impaired endothelial nitric oxide synthase (*eNOS*) protein expression. Human umbilical vein endothelial cells (HUVECs) were pretreated for 2 hours with the indicated concentrations of SLE, followed by oxLDL for 24 hours. For Western blot analyses, a monoclonal anti-eNOS and a monoclonal anti-β-actin antibody (for normalization) were used. The values represent means ± standard error from three separate experiments. *$P < .05$ vs oxLDL treatment.

ROS generation. A previous study demonstrated that oxLDL evokes a progressive rise in cellular ROS, which subsequently leads to the activation of apoptotic signaling.[23] We, therefore, investigated the effects of SLE on the generation of ROS, a potential factor related to oxLDL-induced endothelial cell injury, by using 2,′ 7′-dichlorofluorescein diacetate as a fluorescence probe. Pretreatment of HUVECs with SLE (2.5 to 20 μg/mL) for 2 hours before exposure to oxLDL (130 μg/mL) significantly decreased the level of ROS generation in a dose-dependent manner (all $P < .05$; Fig 2, *A, B*). Intracellular ROS levels are regulated by the balance between ROS generation and antioxidant enzymes. 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 hours; however, SOD-1 was expressed normally after pretreatment with SLE (Fig 2, *C, D*).

Author's personal copy

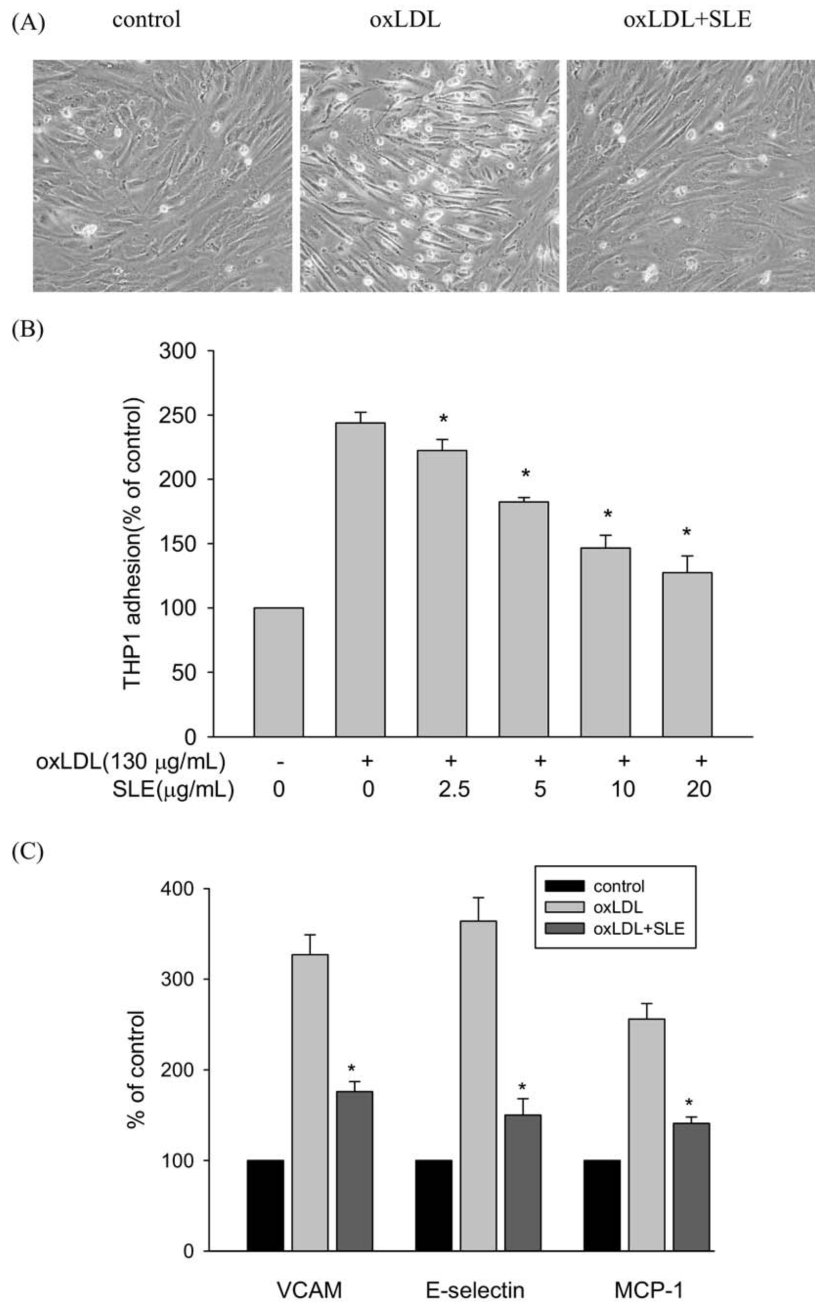

Fig 4. Effect of *Solanum lyratum* extract *(SLE)* on oxidized low-density lipoprotein *(oxLDL)*-induced adhesiveness of human umbilical vein endothelial cells (HUVECs) to THP-1 monocytic cells and adhesion molecule expression. **A,** Representative fields of monocytes adhering to HUVECs with representative treatments as indicated. **B,** Quantification of data for monocyte adherence to endothelial cells incubated with oxLDL (130 μg/mL) for 24 hours or pretreated with indicated concentrations of SLE for 2 hours, followed by treatment with oxLDL. **C,** HUVECs were incubated with oxLDL (130 μg /mL) in the absence or presence of SLE (10 μg/mL) for 24 hours. Histogram of cell surface expression of vascular cell adhesion molecule *(VCAM)*-1, E-selectin, and monocyte chemotactant protein *(MCP-1)* was determined by flow cytometry. The values represent means ± standard error from three separate experiments. *$P < .05$ vs oxLDL treatment.

Expresion of eNOS protein. NO is produced in endothelial cells by the constitutively expressed enzyme eNOS. Considerable evidence indicates that oxLDL-caused endothelial dysfunction is associated with diminished expression of eNOS. We examined eNOS protein expression after treatment with oxLDL. Expression of eNOS proteins was significantly reduced in HUVECs after 24 hours of incubation with oxLDL (Fig 3). Densitometric

Author's personal copy

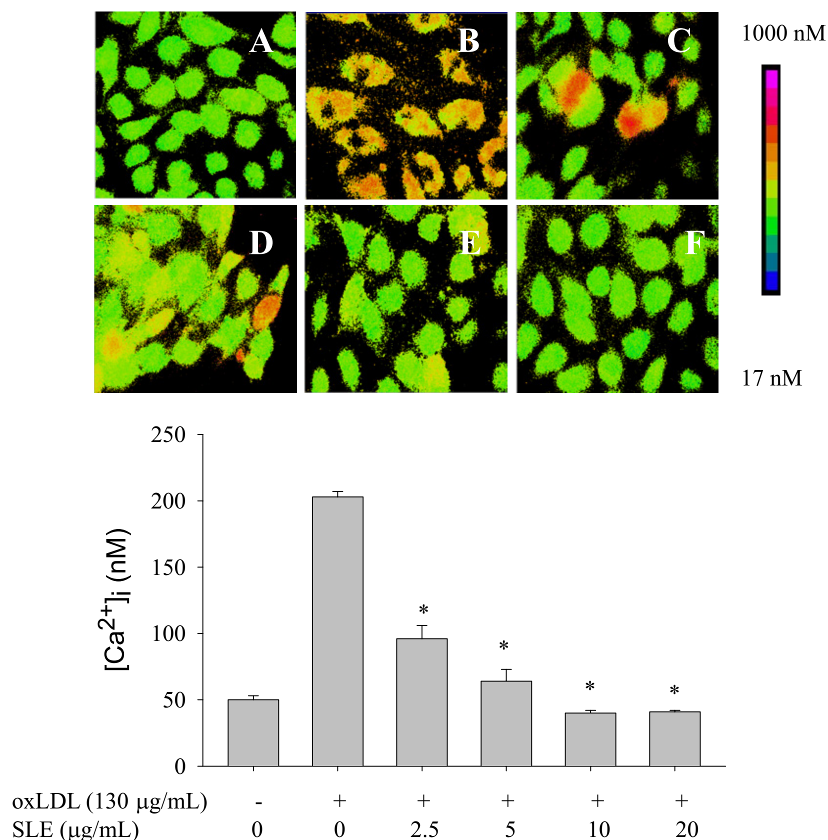

Fig 5. Effect of *Solanum lyratum* extract *(SLE)* on cytoplasmic Ca^{2+} increase stimulated by oxidized low-density lipoprotein *(oxLDL)* in fura 2-loaded human umbilical vein endothelial cells. Images were processed as indicated in Materials and Methods. Calcium changes are color coded *(color bar),* such that warm colors indicate high calcium levels. The values represent means ± standard error of >250 individual cells from three separate experiments. $^*P < .05$ vs oxLDL treatment.

analysis revealed that the level of expression of eNOS in cells treated with SLE (10 μg/mL) before oxLDL was almost that of the control level.

Adherence of THP-1 cells to HUVECs and adhesion molecule expression. OxLDL damages endothelial cells by inducing the expression of adhesion molecules, which subsequently leads to the tethering, activation, and attachment of monocytes to the endothelial cells.[24] To test the effect of SLE on monocyte adhesion to HUVECs, confluent monolayers of HUVECs were pretreated with various concentrations of SLE for 2 hours and then stimulated with oxLDL (130 μg/mL) for 24 hours, followed by incubation with THP-1 cells for 1 hour at 37°C. As shown in Fig 4 (*A* and *B*), oxLDL stimulated the adhesion of THP-1 cells to HUVECs; however, SLE treatment inhibited this adhesion in a dose-dependent manner (2.5 to 20 μg/mL; all $P < .05$). The effect of SLE on the surface expression of adhesion molecules on HUVECs exposed to oxLDL was subsequently examined. Treatment with oxLDL (130 μg/mL) for 24 hours significantly increased VCAM-1, E-selectin, and MCP-1 expression (Fig 4, *C*). Flow cytometry revealed that the induction of adhesion molecule expression was attenuated by 10 μg/mL SLE (all $P < .05$).

Intracellular calcium accumulation. To investigate the effect of chronic exposure of endothelial cells to a cytotoxic concentration of oxLDL on intracellular calcium, we incubated HUVECs with oxLDL (130 μg/mL) in the absence or presence of SLE. The basal $[Ca^{2+}]i$ concentration increased from 50 ± 3nM to 203 ± 4nM in oxLDL-treated cells. SLE significantly inhibited the oxLDL-enhanced rise in intracellular calcium (all $P < .05$; Fig 5).

Mitochondrial transmembrane permeability transition. To examine whether inhibition of mitochondrial disruption accounts for the antiapoptotic effect of SLE, we tested the effects of oxLDL on mitochondrial permeability. When HUVECs were exposed to oxLDL (130 μg/mL), the $^{\Delta}\Psi$m was depolarized, as shown by the increase in green fluorescence (Fig 6, *A*, middle panel). Pretreatment with SLE reduced the change in $^{\Delta}\Psi$m, as indicated by repression of green fluorescence and restoration of red fluorescence (Fig. 6A, right panel). The results from flow cytometry supported these findings. As seen in Fig 6 (*B)* oxLDL caused a marked increase in JC1 green fluorescence (middle) compared with the control (left). Pretreatment with SLE caused marked inhibition of this apoptotic index (right).

Author's personal copy

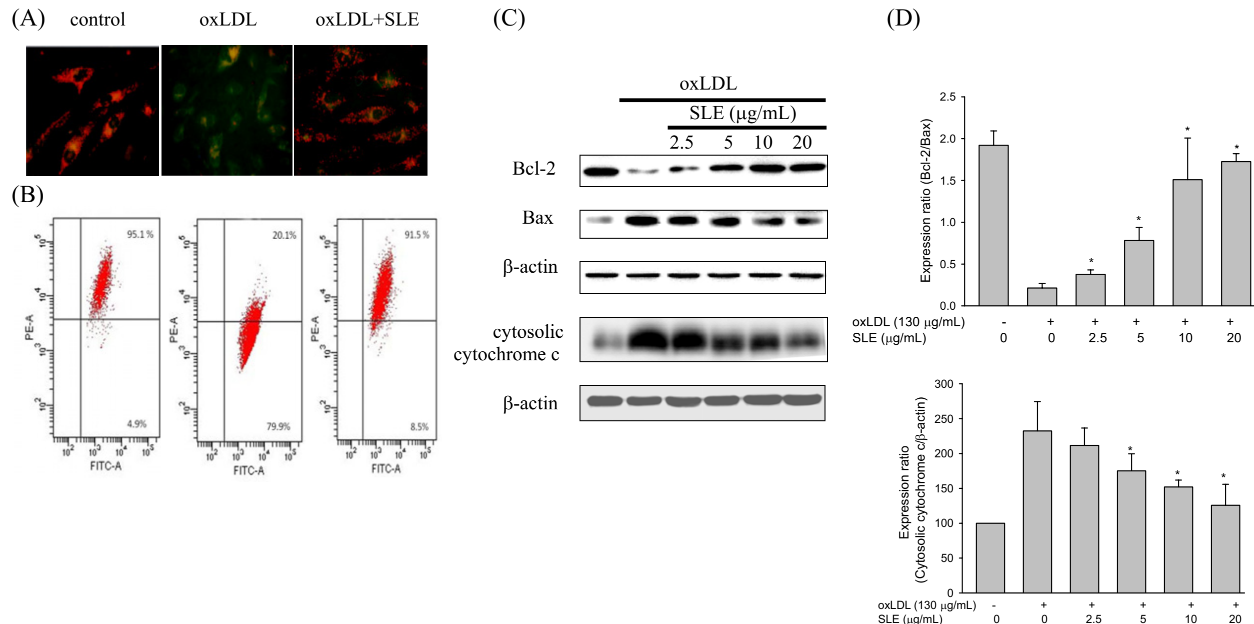

Fig 6. Effect of *Solanum lyratum* extract *(SLE)* on mitochondrial transmembrane permeability transition. **A,** $^{\Delta}\Psi m$ was assessed with the signal from monomeric and J-aggregate JC-1 fluorescence, as described in Materials and Methods: no treatment *(left)*; oxidized low-density lipoprotein *(oxLDL; middle)*; and oxLDL plus SLE *(right)*. **B,** JC-1 fluorescence was confirmed with flow cytometry. **C and D,** Immunoblotting analysis of Bcl-2 protein family and mitochondrial cytochrome *c* release in response to oxLDL and SLE. Human umbilical vein endothelial cells were incubated with oxLDL (130 μg/mL) in the absence or presence of indicated concentrations (2.5-20 μg/mL) of SLE for 24 hours. **C,** Representative Western blots and **(D)** summary data showing oxLDL up-regulated proapoptotic (Bax) and down-regulated antiapoptotic (Bcl-2) proteins *(top)* and increased concentration of cytochrome *c* in the cytosolic fraction *(bottom)*. Pretreatment with SLE suppressed these apoptosis-provoking alterations. Results were subjected to densitometric analysis; the values are presented as means ± standard error of three separate experiments. $^{*}P < .05$ vs oxLDL treatment.

Bcl-2 family proteins are upstream regulators of mitochondrial membrane potential. Because oxLDL depolarized the membrane potential and SLE maintained it, we investigated whether SLE also influenced the equilibrium of Bcl-2 family proteins. Immunoblotting studies demonstrated that oxLDL down-regulated the antiapoptotic (Bcl-2) and up-regulated the proapoptotic (Bax) proteins, whereas SLE pretreatment effectively repressed these oxLDL-evoked proapoptotic events (Fig 6, *C*). Quantitative analysis showed that oxLDL significantly decreased the Bcl-2 to Bax ratio, and that SLE pretreatment preserved this antiapoptotic index (Fig 6, *C* bar graphs).

Disruption of mitochondrial membrane function is known to result in the specific release of the mitochondrial enzyme cytochrome *c* into the cytosol. Therefore, mitochondria were separated from the cytosolic fraction and detected by Western blot. The amount of cytochrome *c* released into the cytosolic fraction was much greater in HUVECs that had been incubated with oxLDL for 24 hours than in control cells (Fig 6, *C*). The results indicate that SLE significantly prevented oxLDL-induced release of cytochrome *c* (Fig 6, *D*).

Activation of caspase-3. Caspase-3 is a key factor in the execution of mitochondrial apoptosis.[20] To examine whether oxLDL and SLE ultimately influence this factor in modulating apoptosis, we determined the active form of caspase-3 by using fluorescence microscopy and flow cytometry. Active caspase-3 was significantly increased in cells that had been treated with oxLDL for 24 hours (Fig 7, *A* and *B*). In contrast, the activation of caspase-3 by oxLDL was suppressed in cells that had been pretreated with 10 μg/mL SLE. The activity of caspase-3 was confirmed by using the EnzCaspase-3 assay kit. The results showed that oxLDL significantly upregulated caspase-3 activity by 3.1-fold, whereas SLE pretreatment effectively suppressed the activity of this apoptotic factor, implying a stimulatory effect of oxLDL and inhibitory action of SLE on caspase-3 activity (Fig 7, *C*, all $P < .05$).

Apoptosis of HUVECs. To further ascertain whether cell death induced by oxLDL was an apoptotic event in HUVECs, oxLDL-treated cells were analyzed biochemically using TUNEL and 4′,6-diamidino-2-phenylindole staining assays and evaluated by microscopic observation and flow cytometry. Cells incubated with oxLDL for 24 hours showed typical features of apoptosis, including the formation of condensed nuclei (Fig 8). These features were not observed in the SLE-pretreated HUVECs. The results of the cell viability assay and the phenotypic features of

Author's personal copy

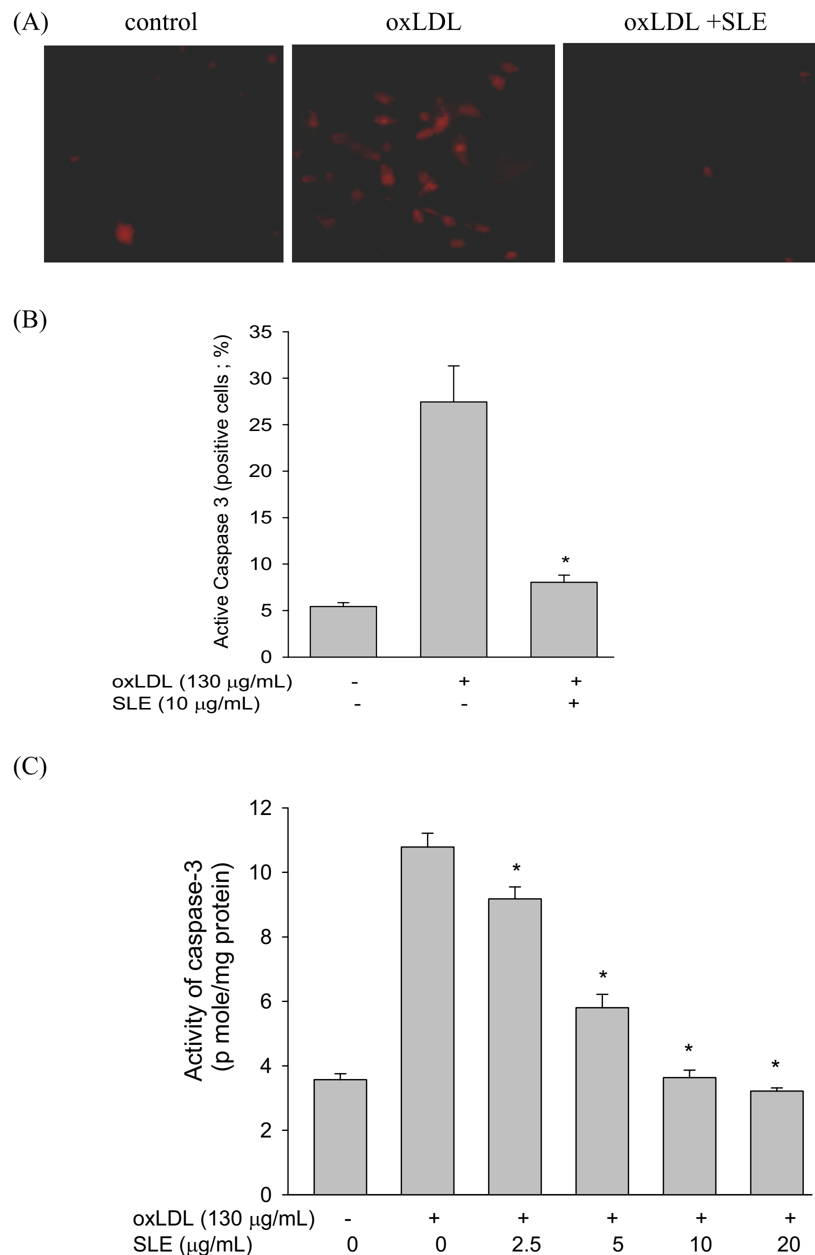

Fig 7. Effects of *Solanum lyratum* extract *(SLE)* on oxidized low-density lipoprotein *(oxLDL)*-induced caspase-3 activation. Human umbilical vein endothelial cells (HUVECs) were incubated with oxLDL in the absence or presence of indicated concentrations of SLE for 24 hours. **A,** Fluorescent images show the activated caspase-3 level in control cells *(left)*, human umbilical vein endothelial cells (HUVECs) stimulated with oxLDL *(middle)*, and in the presence of SLE *(right)*. **B,** Fluorescence intensity of cells was measured with flow cytometry. **C,** The activity of caspase-3 was 3.1-fold higher in oxLDL-treated HUVECs than in the control but was limited to a 2.6-fold to 0.9-fold increase when pretreated with SLE (2.5 to 20 μg/mL). Data are expressed as the mean ± standard error of three independent analyses. $*P < .05$ vs oxLDL treatment.

apoptosis suggest that SLE is a potent inhibitor of the oxLDL-induced cytotoxicity in cultured HUVECs.

DISCUSSION

The oxidant stress imposed by oxLDL appears to activate the signaling pathways leading to apoptosis of endothelial cells. ROS generation is the earliest apoptotic signal of the addition of oxLDL,[1] and then multiple downstream events are activated by secondary messengers.[25] Antioxidants that reduced ROS level inhibited apoptosis, and those that did not reduce ROS level were ineffective.[26] The aim of the present study was to test the hypothesis that SLE

Author's personal copy

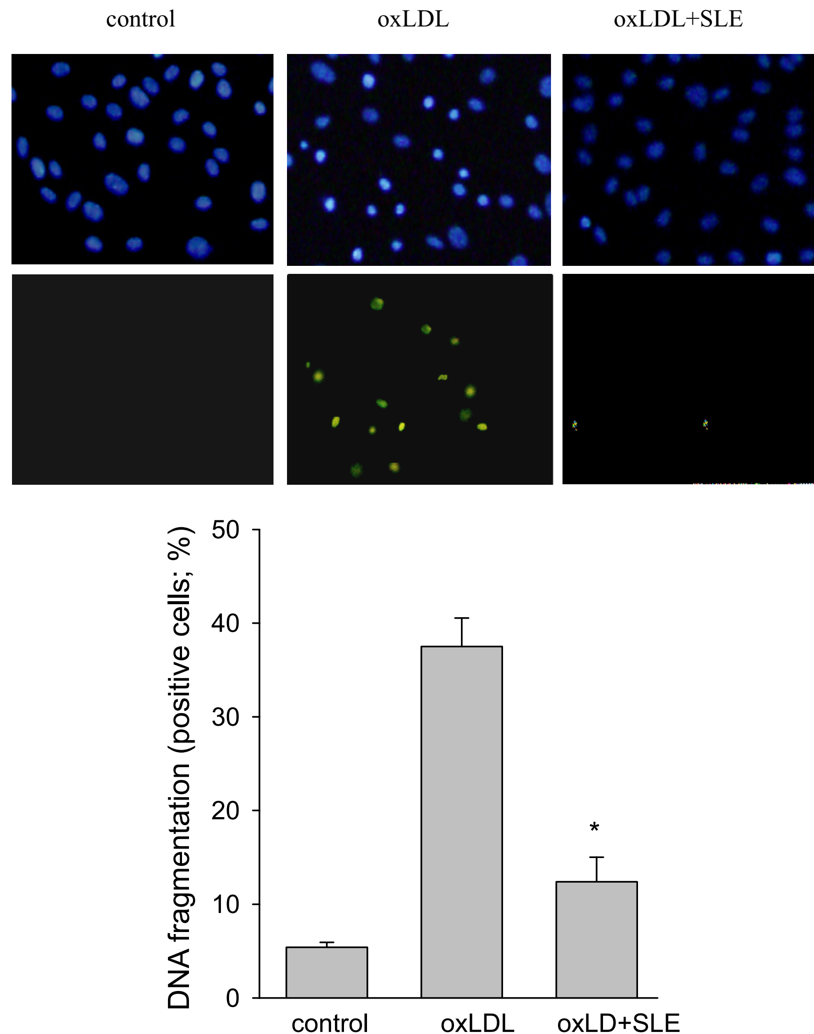

Fig 8. Effect of *Solanum lyratum* extract *(SLE)* on oxidized low-density lipoprotein *(oxLDL)*-induced endothelial cell apoptosis. Human umbilical vein endothelial cells were incubated with oxLDL (130 μg/mL) in the absence *(middle)* and presence *(right)* of 10 μg/mL SLE for 24 hours. **Top,** Cells stained with 4′,6-diamidino-2-phenylindole. **Middle,** Cells stained using terminal deoxynucleotide transferase-mediated deoxy uridine triphosphate nick-end labeling (TUNEL) assay. Fluorescence intensity of cells was measured with flow cytometry. Data are expressed as the mean ± standard error of three independent analyses. $*P < .05$ vs oxLDL treatment.

could protect against oxLDL-induced endothelial dysfunction. Our results revealed that SLE effectively scavenged DPPH radicals and inhibited both copper- and HUVEC-mediated LDL oxidation. Indeed, oxLDL-induced ROS generation was attenuated in the presence of SLE. The reduction in the generation of ROS is likely due to the direct scavenging effect of SLE. This effect subsequently increased the bioavailability of NO, reduced the inflammatory response, maintained the endothelial $[Ca^{2+}]i$ level, and stabilized the mitochondrial membrane potential, thereby preventing the release of cytochrome *c*, a molecule required for the activation of caspase-3 that executes the cell death program. These findings indicate that SLE protects against oxLDL-induced damage by direct antioxidative action.

ROS generated in endothelial cells include superoxide ($\cdot O_2$), hydrogen peroxide (H_2O_2), peroxynitrite ($\cdot$ONOO), NO, and hydroxyl ($\cdot$OH) radicals. Intracellular ROS levels are regulated by the balance between ROS-generating enzymes and antioxidant enzymes that include SOD, catalase, and glutathione peroxidase. SODs protect against superoxide-mediated cytotoxicity by catalyzing $\cdot O_2$ to form H_2O_2; however, SOD-1, but not SOD-2, is inactivated by H_2O_2 formed by dismutation of the superoxide anion.[27] An increase in activity of SOD-1 prevents oxLDL-induced endothelial dysfunction.[28] Our results showed that the effects of SLE on the reduction of ROS preceded the preservation of SOD-1 (2 vs 24 hours), suggesting that the protective effects of SLE against oxLDL-induced endothelial dysfunction could be mainly through the ROS scavenger.

Author's personal copy

NO has pivotal importance in the regulation of vasodilation, leukocyte adhesion, platelet aggregation, and insulin sensitivity. Suppression of NO generation and bioavailability is involved in the development and progression of atherosclerosis as well as the metabolic syndrome.[29] Activated endothelial cells in atheroma can over-express adhesion molecules, which contribute to monocyte recruitment.[30] 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. The antithrombotic and antiatherosclerotic properties of NO are achieved by its ability to inhibit the expression of cell surface adhesion molecules P-selectin, VCAM, and ICAM, and prevent the expression of MCP-1 and inhibit platelet adhesion under flow conditions.[31]

We found that pretreatment with SLE significantly reverses the suppression of eNOS expression by oxLDL, which had an inhibitory effect on the oxLDL-induced adhesiveness between monocytes and HVUECs (Fig 4, *A* and *B*), by a mechanism that is possibly linked to a reduction of cellular superoxide. We next examined the inhibitory effects of SLE on the oxLDL-induced surface expression of adhesion molecules in HUVECs and found that SLE repressed the oxLDL-induced surface expression of these adhesion molecules (Fig 4, *C*). Further work will be necessary to fully define the mechanism of SLE-induced suppression of ROS release from endothelial cells, especially under conditions of treatment with oxLDL.

There is increasing interest in the application of traditional Chinese medicines for the prevention and treatment of cardiovascular disease.[32] One of the reasons is because many phytochemical compounds contained in herbs are potent antioxidants. OxLDL induces the mobilization of Ca^{2+}.[33] Oxygen-derived free radicals and the alteration of intracellular Ca^{2+} ion homeostasis are now considered major contributing factors to atherosclerotic coronary artery disease. ROS increase vascular $[Ca^{2+}]i$ by stimulating inositol trisphosphate-mediated Ca^{2+} mobilization, by increasing cytosolic Ca^{2+} accumulation through sarcoplasmic and endoplasmic reticulum Ca^{2+}-adenosine triphosphatase (ATPase) inhibition, and by stimulating Ca^{2+} influx through Ca^{2+} channels.[20]

Previous studies demonstrated that calcium channel blockers inhibited experimental atherosclerosis in cholesterol-fed animals,[34] improved endothelial cell functions by up-regulating the NO system,[35] down-regulated the endothelial receptor for ox-LDL (LOX-1), and inhibited the CPP32-like protease activity.[36] Antioxidants also prevent both oxLDL-induced ROS generation and Ca^{2+} elevation.[37] ROS such as hydrogen peroxide have been shown to initiate a Ca^{2+} overload in endothelial cells.[38] ROS generation may inhibit Ca^{2+}-ATPases, leading to sustained elevations of $[Ca^{2+}]i$, which are associated with mitochondrial dysfunction through loss of membrane potential and release of cytochrome *c*. Our findings indicate that the antiapoptotic effects of SLE are due to its inhibition of ROS generation, which in turn represses the release of endothelial $[Ca^{2+}]i$ and stabilizes the mitochondrial membrane. This then prevents the release of cytochrome *c*, a molecule required for the activation of caspase-3.

CONCLUSIONS

SLE is a complex extract. Its chemical ingredients include aspidistrin, methylproto-aspidistrin, furostanol glycosides, and steroidal glycosides. To collect sufficient quantities for extensive use as drug remedies, a precursor-feeding strategy to enhance the biosynthesis of pharmaceutical sterols by a cell culture of *Solanum lyratum* has been developed by Lee et al.[39] The present work provides insight into some of the mechanisms in endothelial cells that underlie the vascular protective properties of SLE, highlighting its effects on the reduction of cellular ROS caused by oxLDL. Therefore, SLE may be useful for the prevention of the atherosclerotic process. Our study shows that multiple substances in SLE seem to contribute to its effects. Further investigations on isolation and characterization of the active compounds responsible for the antioxidant capacity of SLE are under way in our laboratory. Our work adds SLE to the growing list of herbal remedies whose mode of action has been at least partially revealed on a molecular level.

AUTHOR CONTRIBUTIONS

Conception and design: WK, CH, JC, SY, SL, HO
Analysis and interpretation: WK, CH, JC, SY, SL, HO
Data collection: WK, KT, TC
Writing the article: WK, CH, SL, HO
Critical revision of the article: SL, HO
Final approval of the article: KT, TC, SL, HO
Statistical analysis: WK, SY, KT
Obtained funding: SL, HO
Overall responsibility: HO
SL and HO contributed equally to this work.

REFERENCES

1. Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, et al. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. J Biol Chem 2000;275:12633-8.
2. Negre-Salvayre A, Alomar Y, Troly M, Salvayre R. Ultraviolet-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cultured cells. III. The protective effect of antioxidants (probucol, catechin, vitamin E) against the cytotoxicity of oxidized LDL occurs in two different ways. Biochim Biophys Acta 1991; 1096:291-300.
3. Galle J, Schneider R, Heinloth A, Wanner C, Galle PR, Conzelmann E, et al. Lp(a) and LDL induce apoptosis in human endothelial cells and in rabbit aorta: role of oxidative stress. Kidney Int 1999;55:1450-61.
4. Lee YY, Hashimoto F, Yahara S, Nohara T, Yoshida N. Steroidal glycosides from Solanum dulcamara. Chem Pharm Bull 1994;42:707-9.
5. Ye W, Wang H, Zhao S, Che C. Steroidal glycoside and glycoalkaloid from Solanum lyratum. Biochem Syst Ecol 2001;29:421-3.
6. Lee YY, Hsu FL, Nohara T. Two new soladulcidine glycosides from Solanum lyratum. Chem Pharm Bull (Tokyo) 1997;45:1381-2.
7. Yahara S MN, Yamasaki M, Hamada T, Kinjo J, Nohara T. A furostanol glucuronide from Solanum lyratum. Phytochemistry 1985;24:2748-50.

Author's personal copy

8. Murakami K, Ezima H, Takaishi Y, Takeda Y, Fujita T, Sato A, et al. Studies on the constituents of Solanum Plants. V. The constituents of Solanum lyratum Thunb. II. Chem Pharm Bull 1985;33:67-73.
9. Kim HM, Lee EJ. Solanum lyratum inhibits anaphylactic reaction and suppresses the expression of L-histidine decarboxylase mRNA. Immunopharmacol Immunotoxicol 1998;20:135-46.
10. Kim HM, Kim MJ, Li E, Lyu YS, Hwang CY, An NH. The nitric oxide-producing properties of Solanum lyratum. J Ethnopharmacol 1999;67:163-9.
11. Hsu SC, Lu JH, Kuo CL, Yang JS, Lin MW, Chen GW, et al. Crude extracts of Solanum lyratum induced cytotoxicity and apoptosis in a human colon adenocarcinoma cell line (colo 205). Anticancer Res 2008;28:1045-54.
12. Wei X, Li CG, Nong S, Zhu XY, Huang XM. [The influence of Solanum lyratum Thunb extract on apoptosis and the expression of fas/fasL genes in Hela cells]. Zhong Yao Cai 2006;29:1203-6.
13. Liu HR, Peng XD, He HB, Wang YH, Li Y, He GX, et al. Antiproliferative activity of the total saponin of Solanum lyratum Thunb in Hela cells by inducing apoptosis. Pharmazie 2008;63:836-42.
14. Shan CM, Li J. Study of apoptosis in human liver cancers. World J Gastroenterol 2002;8:247-52.
15. Kang SY, Sung SH, Park JH, Kim YC. Hepatoprotective activity of scopoletin, a constituent of Solanum lyratum. Arch Pharm Res 1998; 21:718-22.
16. Ou HC, Chou FP, Lin TM, Yang CH, Sheu WH. Protective effects of honokiol against oxidized LDL-induced cytotoxicity and adhesion molecule expression in endothelial cells. Chem Biol Interact 2006;161:1-13.
17. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958;26:1199-1200.
18. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302-10.
19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
20. Sanson M, Ingueneau C, Vindis C, Thiers JC, Glock Y, Rousseau H, et al. Oxygen-regulated protein-150 prevents calcium homeostasis deregulation and apoptosis induced by oxidized LDL in vascular cells. Cell Death Differ 2008;15:1255-65.
21. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440-50.
22. Bedner E, Li X, Gorczyca W, Melamed MR, Darzynkiewicz Z. Analysis of apoptosis by laser scanning cytometry. Cytometry 1999;35:181-95.
23. Schmitt A, Salvayre R, Delchambre J, Negre-Salvayre A. Prevention by alpha-tocopherol and rutin of glutathione and ATP depletion induced by oxidized LDL in cultured endothelial cells. Br J Pharmacol 1995; 116:1985-990.
24. Kim JA, Territo MC, Wayner E, Carlos TM, Parhami F, Smith CW, et al. Partial characterization of leukocyte binding molecules on endothelial cells induced by minimally oxidized LDL. Arterioscler Thromb 1994;14:427-33.
25. Dunn S, Vohra RS, Murphy JE, Homer-Vanniasinkam S, Walker JH, Ponnambalam S. The lectin-like oxidized low-density-lipoprotein receptor: a pro-inflammatory factor in vascular disease. Biochem J 2008; 409:349-55.
26. Hsieh CC, Yen MH, Yen CH, Lau YT. Oxidized low density lipoprotein induces apoptosis via generation of reactive oxygen species in vascular smooth muscle cells. Cardiovasc Res 2001;49:135-45.
27. Jewett SL, Rocklin AM, Ghanevati M, Abel JM, Marach JA. A new look at a time-worn system: oxidation of CuZn-SOD by H_2O_2. Free Radic Biol Med 1999;26:905-18.
28. Tang F, Wu X, Wang T, Wang P, Li R, Zhang H, et al. Tanshinone II A attenuates atherosclerotic calcification in rat model by inhibition of oxidative stress. Vasc Pharmacol 2007;46:427-38.
29. Kurowska EM. Nitric oxide therapies in vascular diseases. Curr Pharm Des 2002;8:155-66.
30. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 2001;107:1255-62.
31. de Graaf JC, Banga JD, Moncada S, Palmer RM, de Groot PG, Sixma JJ. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. Circulation 1992;85:2284-90.
32. Valli G, Giardina E-GV. Benefits, adverse effects and drug interactions of herbal therapies with cardiovascular effects. J Am Coll Cardiol 2002;39:1083-95.
33. Bochkov VN, Mechtcheriakova D, Lucerna M, Huber J, Malli R, Graier WF, et al. Oxidized phospholipids stimulate tissue factor expression in human endothelial cells via activation of ERK/EGR-1 and Ca(++)/NFAT. Blood 2002;99:199-206.
34. Cristofori P, Lanzoni A, Gaviraghi G, Turton J, Sbarbati A. Antiatherosclerotic activity of the calcium antagonist lacidipine in cholesterol-fed hamsters. Biomed Pharmacother 2000;54:93-9.
35. Ding Y, Vaziri ND. Nifedipine and diltiazem but not verapamil upregulate endothelial nitric-oxide synthase expression. J Pharmacol Exp Ther 2000;292:606-9.
36. Sugano M, Tsuchida K, Makino N. Nifedipine prevents apoptosis of endothelial cells induced by oxidized low-density lipoproteins. J Cardiovasc Pharmacol 2002;40:146-52.
37. Maziere C, Morliere P, Massy Z, Kamel S, Louandre C, Conte MA, et al. Oxidized low-density lipoprotein elicits an intracellular calcium rise and increases the binding activity of the transcription factor NFAT. Free Radic Biol Med 2005;38:472-80.
38. Bowles DK, Graier WF, Sturek M. Hydrogen peroxide activates Na(+)-dependent Ca(2+) influx in coronary endothelial cells. Biochem Biophys Res Commun 2001;287:1134-9.
39. Lee MH, Cheng JJ, Lin CY, Chen YJ, Lu MK. Precursor-feeding strategy for the production of solanine, solanidine and solasodine by a cell culture of Solanum lyratum. Process Biochem 2007;42:899-903.

Submitted Mar 2, 2009; accepted Jun 21, 2009.