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Protective Effects of Berberine against Low-Density Lipoprotein (LDL) Oxidation and Oxidized LDL-Induced Cytotoxicity on Endothelial Cells

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The oxidative modification of low-density lipoprotein (LDL) is thought to have a central role in the pathogenesis of atherogenesis. Berberine, a natural constituent of plants of the genera *Coptis* and *Berberis*, has several anti-inflammation and anticancer biological effects. However, its protective effects on LDL oxidation and endothelial injury induced by oxLDL remain unclear. In this study, we evaluated the antioxidative activity of berberine and how berberine rescues human umbilical vein endothelial cells (HUVECs) from oxidized LDL (oxLDL)-mediated dysfunction. The antioxidative activity of berberine was defined by the relative electrophoretic mobility of oxLDL, fragmentation of ApoB, and malondialdehyde production via the Cu^{2+}-mediated oxidation of LDL. Berberine also inhibited the generation of ROS and the subsequent mitochondrial membrane potential collapse, chromosome condensation, cytochrome *C* release, and caspase-3 activation induced by oxLDL in HUVECs. Our results suggest that berberine may protect LDL oxidation and prevent oxLDL-induced cellular dysfunction.

KEYWORDS: Atherogenesis; berberine; oxLDL; HUVECs; ROS

INTRODUCTION

The oxidative modification of low-density lipoprotein (LDL) is a major risk factor for accelerating atherogenesis (*1, 2*). Many studies have demonstrated that oxidized LDL (oxLDL), produced by a variety of different techniques, shows enhanced uptake in macrophages by scavenger receptors and can lead to accumulation of cholesterol ester and formation of foam cells. The accumulation of foam cells in the subendothelial space constitutes a hallmark in atherosclerotic lesion (*3, 4*). The early stages of the atherosclerotic process are initiated by the accumulation of oxLDL and the induction of gene expression in endothelial cells that may result in dysfunction of the endothelium (*5*), leading to an alteration of the function and structural integrity of the endothelial barrier (*6*). It has been reported that oxLDL activates the cellular suicide pathway in endothelial cells, including an increase in reactive oxygen species (ROS) production and a reduction of mitochondrial transmembrane potential with concomitant release of the mitochondrial protein cytochrome *C* and, subsequently, activation of capase-3 and poly ADP-ribose polymerase (PARP), leading to apoptosis (*7*).

Berberine is a bright yellow isoquinoline alkaloid that can be found in the roots, rhizomes, stem, and bark of a number of important medicinal plants, such as *Hydrastis canadensis*, *Cortex phellodendri*, and *Rhizoma coptidis*. The potential clinical importance of berberine has been demonstrated by its use in both Indian and Chinese medical systems (*8*). Berberine has been found to have antibacterial, antibiotic, anti-inflammatory, antioxidant, and immune system-stimulating properties (*9, 10*),

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as well as ameliorating effects on hyperlipidemia and hyperglycemia. A recent report showed that berberine inhibited the progression of diabetes induced by alloxan, and the inhibitory effect might be associated with its hypoglycemic effect, its modulating lipid metabolic effects, and its ability to scavenge free radicals (*11*). It has been well documented that berberine could increase hepatic low-density lipoprotein receptor (LDLR) mRNA and protein levels through a post-transcriptional mechanism (*12*) and inhibit lipid synthesis in human hepatocytes through the activation of AMP kinase (*13*). A placebo-controlled clinical study demonstrated that oral administration of berberine in 32 hypercholesterolemic patients at a daily dose of 1 g for 3 months reduced plasma total triglyceride by 35%, cholesterol by 29%, and LDL cholesterol (LDL-c) by 25% without side effects (*14*). In a previous study, we showed that berberine inhibited the invasion of human lung cancer cells via decreased production of u-PA and MMP-2 (*15*). In addition, the antineoplastic functions of berberine were suggested to be mediated via growth inhibition through cell cycle arrest or the induction of cellular apoptosis (*16*, *17*).

Furthermore, berberine might decrease serum levels of cholesterol, triglycerides, and LDL-cholesterol in hypercholesterolemic patients and in animals fed a high-fat diet (*14*, *18*), but little is known about whether berberine has a protective effect on LDL oxidation and endothelial cells, particularly with regard to oxLDL-induced endothelial dysfunction. In the present study, we investigated the influence of berberine on oxidation of LDL and ox-LDL-induced apoptosis and mitochondrial changes of endothelial cells.

MATERIALS AND METHODS

Materials and Chemicals. Berberine chloride (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in dimethylsulfoxide. Ethylenediaminetetraacetic acid (EDTA), cupric sulfate, sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole (DAPI), and polyacrylamide were purchased from Sigma Chemical Co. (St. Louis, MO). M199, fetal bovine serum (FBS), and trypsin-EDTA were obtained from GibcoBRL (Grand Island, NY). Low serum growth supplement (LSGS) was purchased from Cascade Biologicals (Portland, OR). A monoclonal antibody against β-actin was purchased from Sigma (St. Louis, MO). Mouse monoclonal antibodies against Bcl-2, Bax, and cytochrome *C* and a goat polyclonal antibody against caspase-3 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A rabbit polyclonal antibody against PARP was purchased from BD Transduction Laboratories (San Diego, CA). The ECL Plus detection kit was obtained from Amersham Life Sciences, Inc. (Piscataway, NJ).

Endothelial Cell Cultures. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords with collagenase and used at passages 2–3 (*19*). 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 on every second day.

Lipoprotein Separation and Oxidation. Human plasma was obtained from Taichung Blood Bank, and LDL was isolated using the sequential ultracentrifugation method (*20*). LDL (q = 1.019–1.210 g/mL) in KBr solution containing 30 mM EDTA was stored at 4 °C in a sterile, dark environment and used within 3 days. Immediately before oxidation tests, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 gel (Pharmacia, St-Quentin, France) in 10 mM phosphate-buffered saline (PBS; pH 7.4). LDL was diluted with 10 mM PBS to the final concentration of 1 mg protein/mL and incubated at 37 °C in the presence of $CuSO_4$ (10 μM) for 16 h followed by the removal of Cu^{2+} by gel filtration (PD-10 gel) with 10 mM PBS. Protein quantification was measured by the Bradford protein assay.

Lipid Peroxidation Assay. The malondialdehyde (MDA) production was assessed as an indicator of lipid peroxidation according to the procedures of Camejo et al. (*21*). To each tube containing 0.55 mL of the incubated LDL was added 0.5 mL of 25% (w/v) trichloroacetic acid (TCA) to denature protein. Then, the samples were centrifuged (10000 rpm) at 10 °C for 30 min to remove the pellets. Thiobarbituric acid (TBA; 1%, 0.5 mL) in 0.3% NaOH was added to the supernatant, and the mixed reagents were allowed to react at 90–95 °C for 40 min in the dark. After the reaction was completed, samples were analyzed using a Hitachi F2000 spectrophotofluorimeter (excitation wavelength at 532 nm and emission wavelength at 600 nm). The concentration of MDA or thiobarbituric acid-reacting substance was expressed as equivalents of 1,1,3,3-tetraethoxypropane that served as a standard.

Relative Electrophoretic Mobility (REM) Shift Assays. LDL (200 μg/mL) was pretreated with the indicated concentrations of berberine for 2 h followed by incubation with 10 μM $CuSO_4$ at 37 °C for 16 h. LDL modifications were assessed by agarose electrophoresis to detect the increase in electrophoretic mobility of the modified LDL relative to native LDL accordingly (*22*). In brief, native or modified LDL (8–10 μg) was loaded into 0.6% agarose gels and electrophoresed for 40 min at 100 V. The gel was fixed in 75% ethanol and 5% acetic acid for 15 min, stained with 1% oil red O (in 60% isopropanol) for 30 min, and rinsed with 30% isopropanol to visualize LDL bands. The distance migrated by each LDL band was measured and expressed as an arbitrary REM value compared to the native LDL.

Electrophoresis of ApoB Fragmentation. After the oxidation with or without berberine, samples were denatured with 3% SDS, 10% glycerol, and 5% 2-mercaptoethanol at 95 °C for 5 min. SDS polyacrylamide gel electrophoresis (7.5% SDS-PAGE, 100 V for 6 h) was performed to detect ApoB fragmentation. The gel was subsequently stained with Coomassie brilliant blue R250 and dried (*23*).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay. The DPPH assay was used to measure the antiradical activity of berberine. Briefly, a 0.1 mM DPPH solution was freshly prepared by dissolving DPPH powder in methanol. Then, 10 μL of berberine and Trolox storage solutions were added to 990 μL of the DPPH solution to make the indicated concentrations. The absorbance was measured at 517 nm after 2 min of reaction at room temperature (*24*).

Cell Growth Assay. After treatment with oxLDL (200 μg/mL) for 16 h in the presence or absence of berberine, cells were harvested, and the viable cell number was counted in a hemocytometer by trypan blue exclusion.

Cell Cycle Analysis. HUVECs were pretreated with the indicated concentrations of berberine for 2 h followed by oxLDL (200μg/mL) for 16 h. At the end of the treatment, cells were collected after a brief incubation with trypsin-EDTA and subjected to cell cycle analysis (*25*). Briefly, 1×10^5 cells were fixed in 1 mL of 70% ethanol for 30 min and then suspended in 1 mL of propidium iodide (PI) solution (25 μg/mL PI, 0.1 mM EDTA, and 10 μg/mL RNase A in PBS) for 30 min in the dark. The cell cycle distribution was then analyzed by flow cytometry analysis using Cell-Quest software (Becton-Dickinson, CA).

Measurement of Mitochondrial Membrane Potential. To explore the effect of berberine on the mitochondrial membrane potential ($\Delta\Psi_m$), the lipophilic cationic probe fluorochrome 5,58,6,68-tetraethylbenzimidazolcarbocyanine iodide (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. After treatment with oxLDL (200 μg/mL) for 16 h in the presence or absence of berberine (25, 50, 75, and 100 μM), cells (5×10^4 cells/24-well plate) were rinsed with medium, followed by the addition of JC-1 (5 μM). After an incubation at 37 °C for 20 min, cells were examined under a fluorescent microscope (*26*).

Measurement of ROS Production. ROS have been implicated in the pathophysiology of many vascular disorders. To investigate the effect of berberine on oxLDL-induced ROS production in HUVECs, a

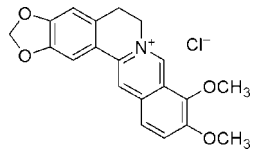

Figure 1. Chemical structure of berberine.

fluorometric assay using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was used as a probe for the presence of hydroxyl radical. Confluent HUVECs were preincubated with berberine for 2 h, and oxLDL (200μg/mL) was then added to the medium in the absence or presence of berberine for 2 h. After the removal of media from wells, cells were incubated with 10 μM DCFH-DA for 1 h. The cells were then centrifuged and resuspended for immediate determination of ROS generation by flow cytometry (Becton-Dickinson, CA) using 488 nm for excitation and 525 nm for emission (*27*).

Determination of Cell Viability (MTT Assay). After the indicated treatments, the treated HUVEC cells were incubated with 0.5 mg/mL MTT in culture medium for an additional 4 h, and the blue formazan crystals of viable cells were dissolved in isopropanol and then measured spectrophotometrically at 563 nm (*28*).

DAPI Staining. Single-cell suspensions of treated HUVEC cells were washed with PBS, fixed with 70% ethanol for 20 min at room temperature, and washed again with PBS. Cells were then treated with DAPI stain (0.6 μg/mL in PBS), incubated for 5 min, and washed again with PBS for 5 min. Chromatin fluorescence was observed under a UV-light microscope. Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and chromatin condensation (*28*).

Western Blot Analysis. Samples of cell lysates or cytosolic fractions were separated in a 12.5% polyacrylamide gel and transferred onto a nitrocellulose membrane as previously described (*29*). The blot was subsequently probed with β-Actin, Bax, Bcl-2, cytochrome *C*, caspase 3, and PARP antibodies. The immunoreactions were detected by chemiluminescence using an an ECL Plus detection kit, and the relative intensities of the signals were quantified by densitometry using a gel documentation and analysis system (LAS-3000 Image Reader, Fujifilm, Stamford, CT).

Statistical Analysis. An unpaired two-tailed Student *t* test was used to assess group differences throughout this study (SigmaStat 2.0, Jandel Scientific, San Rafael, CA).

RESULTS

Berberine Inhibited the Copper-Induced Oxidation of LDL and ApoB Fragmentation. The chemical structure of berberine is illustrated in **Figure 1**. The effects of berberine on $CuSO_4$-mediated oxidation of LDL were determined by REM assay. LDL was incubated with 10 μM $CuSO_4$ for 16 h to induce oxidation of LDL. As shown in **Figure 2A** and **2B**, the REM of the control was assigned a mobility of 1.0 while chemical oxidation shifted the REM to 4.2. Pretreatment of berberine caused a such significant and dose-dependent reduction of electrophoretic mobility that the REM was lowered to 2.8, 1.8, 1.5, and 1.3, respectively, after pretreatment with 25, 50, 75, and 100 μM berberine. In addition, after the treatment with 10, 50, and 100 μM Trolox, a water-soluble analogue of vitamin E (as a positive control), the REM was reduced to 2.5, 1.2, and 1.1, respectively. The inhibitory effects of berberine on ApoB fragmentation were also examined by SDS-PAGE in a similar manner (**Figure 2C**). The ApoB band (arrow) in native LDL disappeared due to the fragmentation caused by the incubatation with $CuSO_4$ for 4 h. The presence of berberine (25, 50, 75, and 100 μM) and Trolox (10, 50, and 100 μM) significantly reversed the copper-induced ApoB fragmentation (**Figure 2C**). The signal

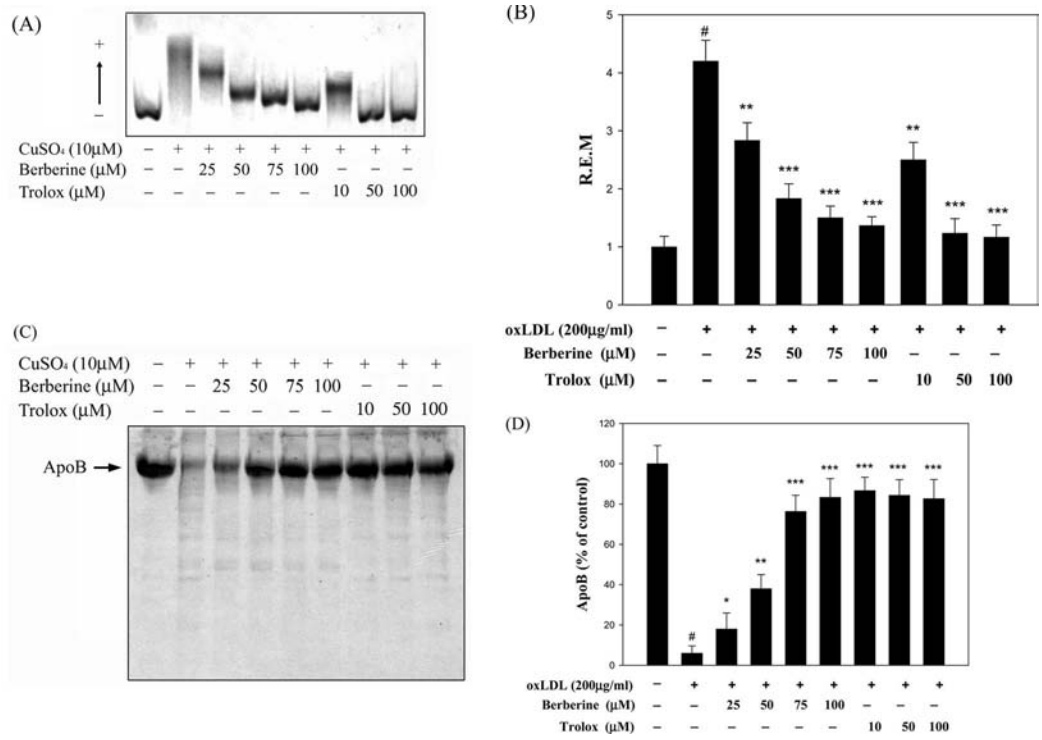

Figure 2. Effect of berberine on the Cu^{2+}-mediated shift of electrophoretic mobility and the ApoB fragmentation in LDL. (**A**) LDL was incubated with 10 μM $CuSO_4$ for 16 h at 37 °C in the presence or absence of berberine or Trolox, as positive control, and applied to 0.6% agarose gels as described in the Materials and Methods. (**B**) The results from the agarose gel electrophoresis were quantified and expressed in the form of relative electrophoretic mobility (REM). The distance traveled in the agarose gel by the native LDL was assigned the arbitrary unit 1. (**C**) LDL (200 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 °C in the absence or presence of berberine or Trolox for 4 h and applied to 7.5% SDS-PAGE as described in the Materials and Methods. (**D**) Quantification of the ApoB fragmentation assay using densitometry is presented as means ± SD of three independent experiments. The signal intensity of the native LDL was assigned as 100% arbitrarily. #, $P < 0.001$ compared with control. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with the oxLDL-treated group.

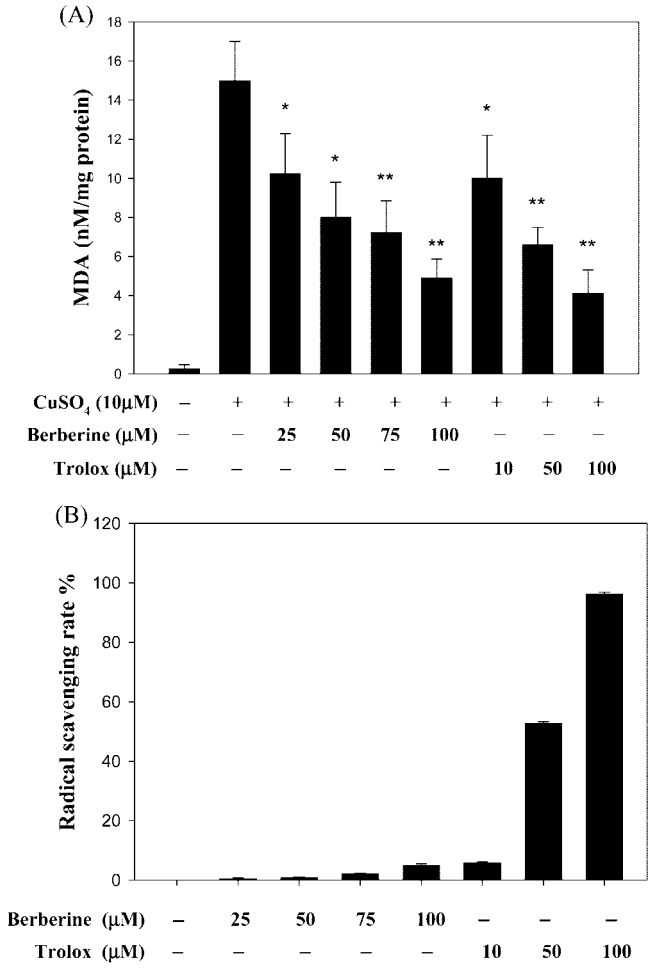

Figure 3. Effect of berberine on Cu^{2+}-induced MDA formation of LDL and DPPH scavenging capability. (**A**) LDL (200 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 °C for 16 h in the presence or absence of berberine or Trolox, and then MDA formation was measured as described in the Materials and Methods. (**B**) The radical-scavenging activities of berberine and Trolox were evaluated by using the DPPH radical scavenging assay. The absorbance of the sample without adding berberine or Trolox was assigned as 100%, and its radical scavenging rate was assigned as 0% consequently. The quantitative data were presented as means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with the oxidative LDL-treated group.

intensities of ApoB present in LDL samples under different treatments were quantified and compared densitometrically (**Figure 2D**).

Berberine Attenuated Copper-Induced Lipid Peroxidation of LDL. The degree of LDL oxidation was assessed by measuring the concentration of malondialdehyde (MDA), as a lipid peroxidation indicator. **Figure 3A** shows a significant increase in the MDA production when LDL was incubated with 10 μM $CuSO_4$ (lane 2, **Figure 3A**). In contrast, berberine (25, 50, 75, and 100 μM) or Trolox (as a positive control; 10, 50, and 100 μM) significantly reduced the MDA formation in a dose-dependent manner, indicating that berberine exerted an inhibitory effect on lipid peroxidation of LDL. Since berberine exhibited a protective effect in LDL oxidation, we further investigated the radical scavenging activity of berberine. In order to assess the radical scavenging potential of berberine, the reactivity toward the stable free radical DPPH was measured at 517 nm. As shown in **Figure 3B**, berberine demonstrated little or marginal activity when compared to Trolox, which appeared to be a more effective radical scavenger.

Berberine Reduced the OxLDL-Induced Cytotoxicity on HUVECs. The protective effect of berberine was examined by monitoring the oxLDL-induced morphological features of cultured HUVECs in the absence or presence of berberine under phase-contrast microscopy. Treatment of cultured endothelial cells with oxLDL (200 μg/mL) for 16 h led to cell shrinkage or membrane blebbing, which were notably reduced by adding berberine and Trolox (**Figure 4A**). This observation was further supported by berberine-induced increment of cell viability (**Figure 4B**) and proliferative activities (**Figure 4C**) in oxLDL-treated HUVECs.

To investigate whether berberine conferred protection against oxLDL-induced apoptosis in HUVECs, DAPI staining and flow cytometry analysis were performed. The cells incubated with oxLDL for 16 h showed the typical features of apoptosis, the formation of condensed chromatin (**Figure 5A**), and the accumulation of a sub-G1 population (**Figure 5B**), while this effect was significantly decreased by berberine and Trolox pretreatment. Together, these suggested that berberine protected endothelial cells from oxLDL-induced cytotoxicity and apoptosis.

Berberine Perturbed oxLDL-Induced ROS Generation in HUVECs. The effects of berberine on oxLDL-mediated ROS generation were determined by pretreating endothelial cells with berberine (2 h) before oxLDL exposure. As shown in **Figure 6**, oxLDL (200μg/mL) produced approximately a 5-fold increase in ROS generation (**Figure 6B**, dark trace) as compared to the control (**Figure 6A**, gray trace). Pretreatment with berberine (25, 50, and 75 μM) significantly inhibited (left shift) the oxLDL-induced ROS formation (**Figure 6C–E**), while 100 μM berberine almost completely abolished the inhibited ROS production (**Figure 6F**).

Berberine Reverted OxLDL-Induced Alterations in Mitochondrial Transmembrane Permeability. To test whether inhibition of mitochondrial disruption was involved in the antiapoptotic effect of berberine, we examined the effects of oxLDL on mitochondrial permeability. When HUVECs were exposed to oxLDL, the $\Delta\Psi_m$ of mitochondria was depolarized, as shown by the decrease in red fluorescence and the increase in green fluorescence. Pretreatment with berberine reduced the change in $\Delta\Psi_m$, as indicated by the repression of green fluorescence and the restoration of red fluorescence (**Figure 7**). The disruption of mitochondrial membrane function is known to result in the release of the mitochondrial enzyme cytochrome *C* into the cytosol, which can be detected by Western blot analysis. The incubation of HUVECs with oxLDL for 16 h induced a 3-fold increase in the release of cytochrome *C* into the cytosolic fraction, compared with the case of the control cells (**Figure 8A**). Notably, berberine significantly reduced the oxLDL-induced release of cytochrome *C*.

Berberine Prevented the OxLDL-Induced Changes in Apoptotic Protein Expression in HUVECs. It is recognized that proteins of the Bcl-2 family have crucial roles in the regulation of apoptosis by functioning as promoters (e.g., Bax) or inhibitors (e.g., Bcl-2) of cell death, and oxLDL-induced release of cytochrome *C* may lead to the activation of caspase-3 and the cleavage of PARP. The effect of berberine on the expression of these proteins in HUVECs was determined by Western blot analysis. Berberine reduced the oxLDL-induced expression of Bax significantly and increased the levels of antiapoptotic protein Bcl-2 in a dose-dependent manner (**Figure**

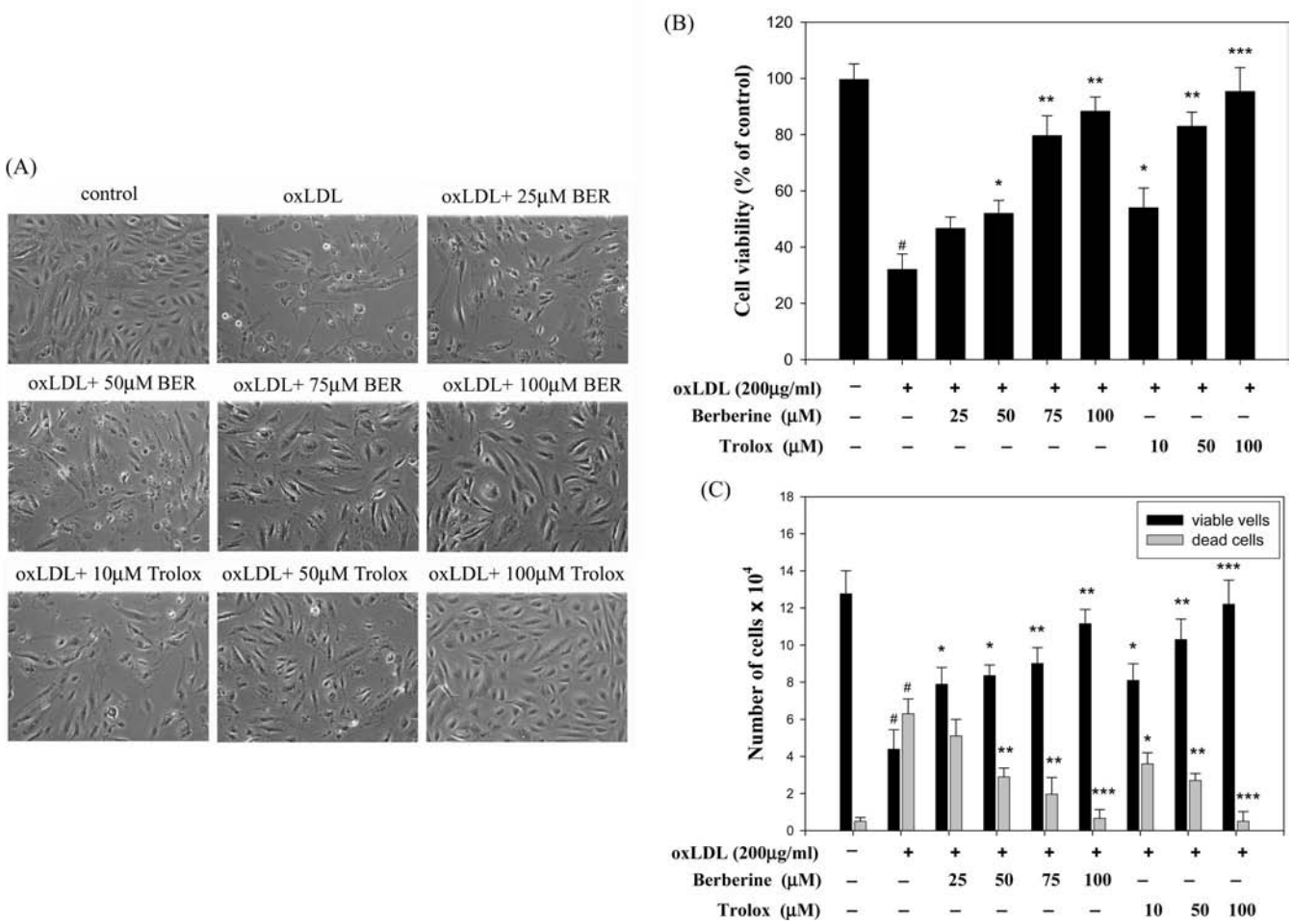

Figure 4. Effect of berberine on oxLDL-induced endothelial cell death. HUVECs were incubated with oxLDL (200 μg/mL) in the absence and presence of berberine (BER; 25, 50, 75, and 100 μM) or Trolox (10, 50, and 100 μM) for 16 h. (**A**) Photomicrographs of the treated HUVEC cells were observed by using phase-contrast microscopy. (**B**) The viability of treated HUVEC cells was detected using the MTT assay as described in the Materials and Methods. (**C**) Viable cells and dead cells were counted using the Trypan blue exclusion assay. The quantitative data were presented as means ± SD of three independent experiments. #, $P < 0.001$ compared with control. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with the oxLDL-treated group.

8B). The expression levels of active caspase-3 and cleaved-PARP were increased significantly in HUVECs treated with oxLDL. In contrast, the activation of caspase-3 and the cleavage of PARP by oxLDL were suppressed dose-dependently in cells pretreated with berberine.

DISCUSSION

Earlier reports indicated that oxidation of the LDL–lipid moiety, which leads to the generation of lipid peroxidation products such as thiobarbituric acid-reacting substances, is thought to represent the initial step of oxidative pathway modification (*30*, *31*). Large amounts of lipid peroxides accompanied by rearrangement of fatty acid double bonds yielded conjugated dienes that induced cholesterol degradation. As a consequence of the propagation reactions, fatty acid fragmentation occurs, leading to the formation of highly reactive intermediates, such as ketones and aldehydes, which can then form a complex with the adjacent ApoB, the major protein within LDL, to induce fragmentation of ApoB (*3*, *32*, *33*). Since oxLDL represents a major clinical risk factor for atherosclerosis, reducing the formation of oxLDL is of a great importance in the prevention of heart diseases. The present data demonstrate that berberine partially prevents the increase in REM (**Figure 2A**) and MDA formation (**Figure 3A**) in response to the incubation of LDL with Cu^{2+}, indicating that it exerts an important antioxidant effect in this system. Although the MDA production was not completely reversed by berberine, i.e. to the basal levels seen in the absence of Cu^{2+}, it is likely that oxidative stress was relieved to a sufficient degree to allow normalization of ApoB fragmentation (**Figure 2C**). These data suggest that berberine possesses a strong antioxidative ability in inhibiting LDL oxidation.

An elevated level of serum LDL cholesterol is an essential factor in the pathogenesis of atherogenesis (*34*). The endothelium plays a key role in many cardiovascular diseases, and apoptosis is emerging as a determinant process in the disease progress (*35*). It has been suggested that a substantial accumulation of LDL in the arterial wall, where it becomes oxLDL and impairs endothelial function in hypercholesterolemic patients, is a monumental event in early atherogenesis (*36*). LDL undergoes oxidative modification when incubated with endothelial cells, macrophages, or smooth muscle cells, and oxidized LDL is taken up rapidly by macrophages via scavenger receptors, transforming them into foam cells, which are essential components of fibrofatty plaques and fatty streaks. Accumulation of foam cells in the subendothelial space is a hallmark of atherosclerotic lesion (*6*, *37*, *38*). In the present study, berberine displayed a significant ability in the prevention of oxidative

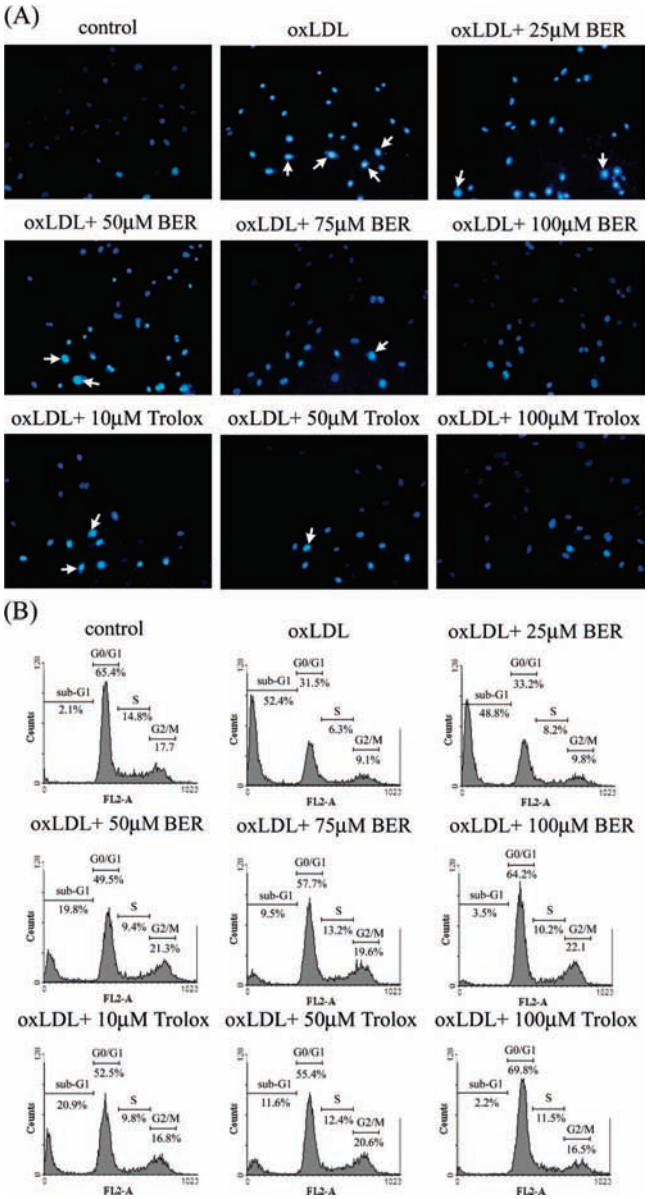

Figure 5. Protective effect of berberine on oxLDL-induced endothelial cell apoptosis. HUVECs were incubated with oxLDL in the presence and absence of berberine (BER) or Trolox for 16 h. (**A**) The nuclear morphology of the treated cells was observed by fluorescence microscopy using DAPI stain (at a magnification of 200×). Arrows showed areas of intense fluorescence staining with condensed nuclei. (**B**) The hypodiploid cell population (sub G1 phase) of the treated HUVEC cells was analyzed by flow cytometry using PI stain, and at last, 10000 events of total cells were analyzed for each experimental treatment.

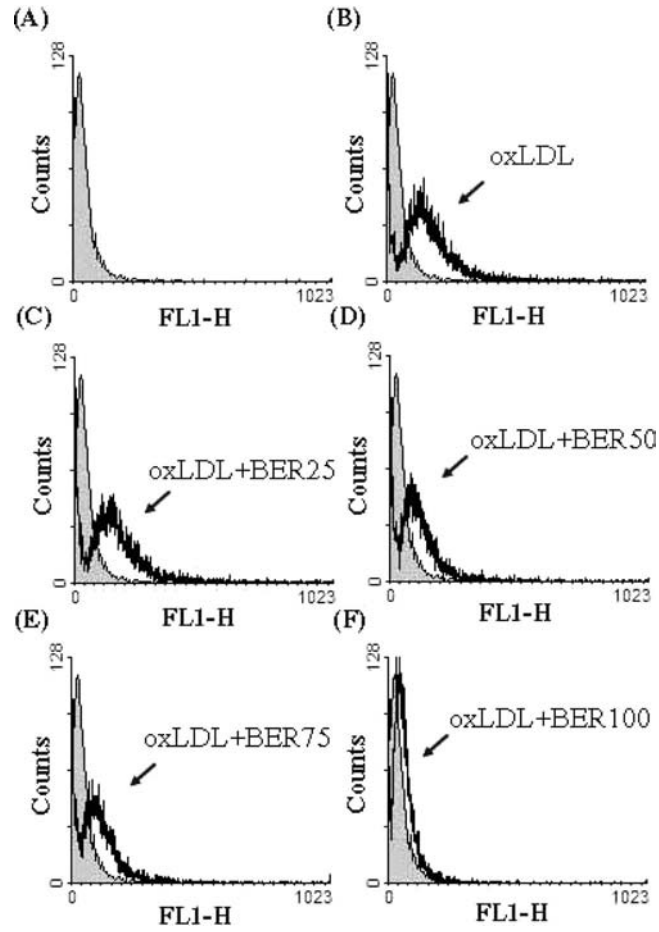

Figure 6. Effects of berberine on oxLDL-induced ROS production in HUVECs. The ROS levels of (**A**) the HUVEC cells without treatment, control (gray trace); (**B**) the HUVEC cells with treatment of 200 μg/mL oxLDL, oxLDL (dark trace); (**C**) the HUVEC cells with treatment of oxLDL and 25 μM berberine, oxLDL+BER25 (dark trace); (**D**) the HUVEC cells with treatment of oxLDL and 50 μM berberine, oxLDL+BER50 (dark trace); (**E**) the HUVEC cells with treatment of oxLDL and 75 μM berberine, oxLDL+BER75 (dark trace); and (F) the HUVEC cells with treatment of oxLDL and 100 μM berberine, oxLDL+BER100 (dark trace), were measured by flow cytometry using DCFH staining.

damage to the endothelial cells *in vitro* (**Figures 4** and **5**). This protective effect by berberine is concomitant with a decrease in levels of ROS (**Figure 6**) and the stabilization of mitochondrial integrity (**Figure 7**). Consequently, the levels of mitochondrial cytochrome *C* (**Figure 8A**) and cleaved apoptotic effectors, caspase 3 and PARP (**Figure 8B**), were significantly down-regulated, implying a reduced extent of apoptosis. Collectively, berberine could protect endothelial cells against oxLDL-induced apoptosis and provide an antiatherosclerotic effect through cytochrome *C*-mediated pathways. In addition, the pharmacokinetic property of oral absorption of 1.2 g of berberine has been reported for healthy volunteers. The *T* (peak) was 2.37 ± 0.04 h, and the *C* (max) was 394.7 ± 155.4 ng/mL (*39*). The plasma concentration of berberine responding to oral administration and intravenous injection in rat has also been well established to show that the plasma concentration of berberine reached 1.81 μg/mL after injection of 10 mg/kg berberine through the femoral vein and reached 2.31 μg/mL after oral administration of 27.6 mg/kg berberine (*40, 41*). Berberine was eliminated very slowly in the rat body, and the lowest observed adverse effect level (LOAEL) remained at 531 mg/(kg day) (*42*). In this study, we showed that 25 μM berberine protected HUVEC cells from oxLDL-induced cytotoxicity. Based on its well-known pharmacokinetics and the efficiency level tested *in vitro*, we consider that berberine would be a suitable candidate applied to a relative *in vivo* study.

Berberine, a quaternary protoberberine-type alkaloid, has a dibenzo[*a*,*g*]quinolizidine ring system structure (**Figure 1**). The previous studies assume that a polar function including a nitrogen atom in the center as well as a dimethoxybenzo group and a [1,3]dioxolobenzo group at both its ends would be a pharmacophore of alkaloids (*43*). Similarly to phenolic antioxidants, the presence of an aromatic hydroxyl group may be responsible for the antioxidant efficiency of alkaloids (*44*). However, berberine lacks an aromatic hydroxyl group in its chemical structure and showed only a negligible activity in

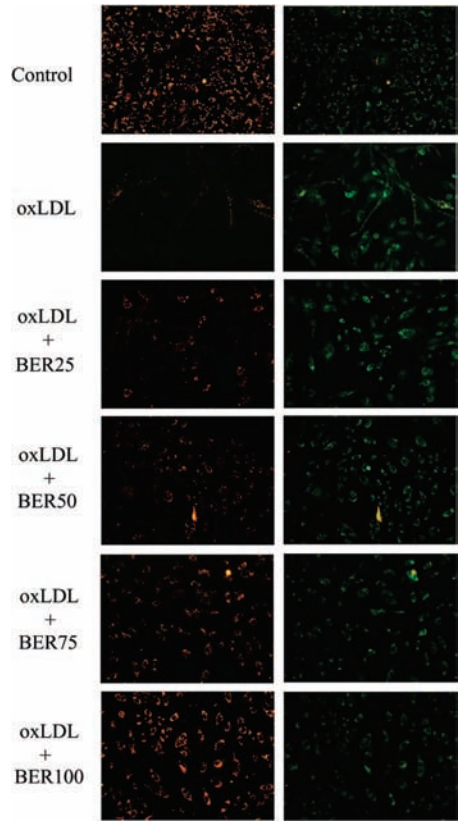

Figure 7. Effects of berberine on oxLDL-induced changes in the mitochondrial membrane potential in endothelial cells. HUVECs were incubated with oxLDL (200 μg/mL) in the absence and presence of berberine (BER; 25, 50, 75, and 100 μM) for 16 h. The changes of the mitochondrial membrane potential ($\Delta\Psi_m$) were assessed by using fluorescent lipophilic cationic JC-1 dye. JC-1 is selectively accumulated within intact mitochondria to form multimer *J*-aggregates emitting fluorescence light at 590 nm (red) at a higher membrane potential, *left*, and monomeric JC-1 emits light at 527 nm (green) at a low membrane potential, *right*.

radical scavenging (**Figure 3B**). By using Trolox as a reference antioxidant, berberine showed a similar effect on the prevention of LDL oxidation (**Figures 2** and **3**) and oxLDL-induced endothelial injury (**Figures 4** and **5**). Moreover, berberine exhibited a strong inhibition of oxLDL-induced ROS overproduction (**Figure 6**). It has been reported that berberine exhibited protective effects against oxidative damage by reducing cellular peroxynitrite ($ONOO^-$) formation, reversing the suppression of antioxidant enzymes (SOD, catalase, glutathione peroxidase), inhibiting the oxidative metabolic activities of cytochrome P450, and suppressing iNOS expression (*45*, *46*). Other studies indicated that berberine inhibited the expression of several proteins, such as IL-1β, TNFα, and HIF-1α, through transcriptional regulation or proteolytic degradation (*47*, *48*). Thus, we speculated that the protection of berberine against oxLDL-induced endothelial dysfunction might be associated with suppression of ROS overproduction, and this effect might be through modulating the expression or activity of ROS generating enzyme and ROS scavenging enzyme, as opposed to directly scavenging ROS activity attributed to its chemical structure. However, the exact mechanism of action by which berberine exerts protection against oxidative stress remains to be elucidated and requires further examination.

Our results suggest a possible mechanism linking ROS generation, membrane damage, and induction of apoptosis to the damage caused by oxLDL in HUVECs. Further understanding of the intracellular signaling pathways modulating the oxidative damage induced by oxLDL in the endothelial cultures may enable us to devise potential therapeutic tools for the prevention of atherosclerotic progression.

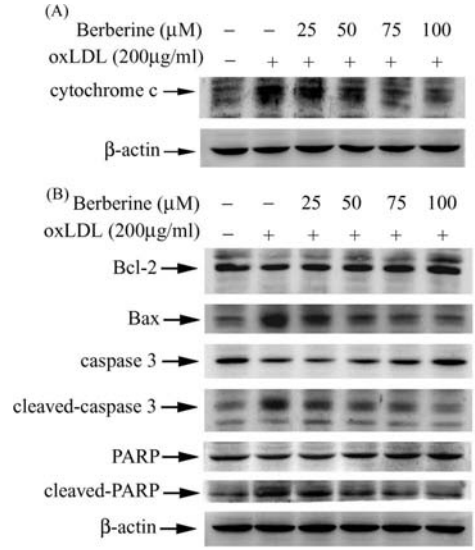

Figure 8. Effects of berberine on oxLDL-induced caspase-3 and PARP activation. In the Western blot assay, cell lysates were subjected to SDS-PAGE, with β-Actin used as an internal control. Signals of proteins were visualized with an ECL detection system. The results were representative of three independent experiments.

ABBREVIATIONS USED

LDL, low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; oxLDL, oxidized low-density lipoprotein; DAPI, 4′,6-diamidino-2-phenylindole; REM, relative electrophoretic mobility; ROS, reactive oxygen species; PARP, poly ADP-ribose polymerase.

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