Ginkgo biloba Extract Inhibits Tumor Necrosis Factor-α–Induced Reactive Oxygen Species Generation, Transcription Factor Activation, and Cell Adhesion Molecule Expression in Human Aortic Endothelial Cells

Jaw-Wen Chen, Yung-Hsiang Chen, Feng-Yan Lin, Yuh-Lien Chen, Shing-Jong Lin

Objective—This study was conducted to examination whether Ginkgo biloba extract (GBE), a Chinese herb with antioxidant activity, could reduce cytokine-induced monocyte/human aortic endothelial cell (HAEC) interaction, a pivotal early event in atherogenesis.

Methods and Results—Pretreatment of HAECs with GBE (50 and 100 μg/mL for 18 hours) significantly suppressed cellular binding between the human monocyctic cell line U937 and tumor necrosis factor-α (TNF-α)-stimulated HAECs by using in vitro binding assay (68.7% and 60.1% inhibitions, respectively). Cell enzyme–linked immunosorbent assay and immunoblot analysis showed that GBE (50 μg/mL for 18 hours) significantly attenuated TNF-α–induced cell surface and total protein expression of vascular cellular adhesion molecule-1 and intracellular adhesion molecule-1 (63.5% and 69.2%, respectively; P<0.05). However, pretreatment with probucol (5 μmol/L for 18 hours) reduced the expression of vascular cellular adhesion molecule-1 but not intracellular adhesion molecule-1. Preincubation of HAECs with GBE or probucol significantly reduced intracellular reactive oxygen species formation induced by TNF-α (76.8% and 68.2% inhibitions, respectively; P<0.05). Electrophoretic mobility shift assay demonstrated that both GBE and probucol inhibited transcription factor nuclear factor-κB activation in TNF-α–stimulated HAECs (55.2% and 65.6% inhibitions, respectively) but only GBE could inhibit the TNF-α–stimulated activator protein 1 activation (45.1% inhibition, P<0.05).

Conclusions—GBE could reduce cytokine-stimulated endothelial adhesiveness by downregulating intracellular reactive oxygen species formation, nuclear factor-κB and activator protein 1 activation, and adhesion molecule expression in HAECs, supporting the notion that the natural compound Ginkgo biloba may have potential implications in clinical atherosclerosis disease.

Key Words: activator protein 1 ■ cell adhesion molecule ■ Ginkgo biloba ■ human aortic endothelial cells ■ nuclear factor-κB

Ginkgo biloba (Chinese name, Pai-kuo or Yin-hsing), a Chinese herb, has been used in traditional Chinese medicine for thousands of years.1 Recently, Ginkgo biloba extract (GBE), a defined complex mixture containing 24% Ginkgo flavone glycoside and 6% terpenlactones (ginkgolides, bilobalide) that is extracted from Ginkgo biloba leaves, has been used as a therapeutic agent for some cardiovascular and neurological disorders, especially dementia.1,2 Although the exact mechanism is not known, accumulating in vitro and in vivo evidence demonstrated the protective effects of GBE in ischemia/reperfusion injury3 and under increased oxidative stress.4 It is then suggested that the pharmacological effects of GBE are closely related to its antioxidant ability to scavenge free radicals.5–7

Atherosclerosis is a chronic inflammatory process with increased oxidative stress in which the adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall are the pivotal early events in atherogenesis.8,9 The interaction between monocytes and vascular endothelial cells could be mediated by adhesion molecules including vascular adhesion molecule 1 (VCAM-1),10 intercellular adhesion molecule 1 (ICAM-1),11 and E-selectin12 on the surface of the vascular endothelium. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) could activate nuclear factor-κB (NF-κB)13,14 and activator protein-1 (AP-1),15–17 the 2 major redox-sensitive eukaryotic transcription factors that regulate genes relevant to the expression of adhesion molecules.18,19 Because the acti-
vation of NF-κB or AP-1 could be inhibited to various degrees by different antioxidants, it is strongly suggested that endogenous reactive oxygen species (ROS) may play an important role in these redox-sensitive transcription pathways in atherosclerosis.18,19

Although GBE was shown with antioxidant effect both in vivo and in vitro, whether it can directly inhibit atherosclerosis has not been clarified. In this study, we tested the hypothesis that GBE could inhibit cytokine-induced endothelial adhesion to monocytes by downregulating endogenous ROS production, redox-sensitive transcription pathways, and the expression of adhesion molecules in vascular endothelial cells. The results may provide rationales for potential implications of GBE in clinical atherosclerosis vascular disease.

Methods

Cell Cultures

Human aortic endothelial cells (HAECs, Cascade Biologics) were grown in Medium 200 (Cascade Biologics) supplemented with low serum growth supplement (Cascade Biologics) in an atmosphere of 95% air and 5% CO₂ at 37 °C in plastic flasks. The final concentrations of the components in Medium 200 contained 2% FBS (Gibco-BRL), 1 µg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/mL human fibroblast growth factor, 10 µg/mL heparin, and 1% antibiotic-antimycotic mixture (GibcoBRL). At confluence, the cells were subcultured at a 1:3 ratio and used at passage numbers 3 through 8. The human monocytic cell line U937 (American Type Culture Collection) was grown in suspension culture in RPMI-1640 (GibcoBRL) containing 10% FBS and 1% antibiotic-antimycotic mixture (GibcoBRL). At confluence, the cells were subcultured at a 1:3 ratio. After incubation with GBE (kindly provided by Dr Willmar Schwabe), probucol (Sigma), or TNF-α, cell viability was always greater than 90% by using trypan blue exclusion method or MTT assay.

Monocyte-Endothelial Cell Adhesion Assay

To explore the effect of GBE on endothelial cell–leukocyte interaction, the adherence of U937, a human monocytic cell line, to TNF-α-activated HAECs was examined under static conditions. HAECs were grown to confluence in 24-well plates and preincubated with GBE or probucol for 18 hours and stimulated with TNF-α for 6 hours. The adhesion assays were then performed as previously described, with minor modification.20 Briefly, U937 cells were labeled with fluorescent dye, incubated with 10 µmol/L 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Molecular Probes) at 37°C for 1 hour in RPMI-1640 medium, and subsequently washed by centrifugation. Confluent HAECs in 24-well plates were incubated with U937 cells (10⁶ cells/mL) at 37°C for 1 hour. Nonadherent leukocytes were removed, and plates were gently washed twice with PBS. The numbers of adherent leukocytes were determined by counting 4 fields per 100 high-power-field well using fluorescent microscopy (Nikon) and photographed. Four randomly chosen high-power fields were counted per well. Experiments were performed in duplicate or triplicate and were repeated at least 3 times.

Cell Enzyme–Linked Immunosorbent Assay

To examine whether GBE could modify the expression of VCAM-1, ICAM-1, and E-selectin, cell ELISA was conducted. The expression of ICAM-1, VCAM-1, and E-selectin on HAECs surface was quantified as previously described.21 Briefly, at 95% confluence in 96-well microplates, antioxidants were added to HAECs 18 hours before activation or during the 6-hour TNF-α activation period. The monolayers were washed and then incubated with goat anti-human ICAM-1, VCAM-1, or E-selectin monoclonal antibodies (R&D Systems) at a final concentration of 0.5 µg/mL in HBSS containing 1% skim milk to detect the surface expression of these adhesion molecules. After incubation of cells at room temperature for 30 minutes, the plates were washed 4 times with HBSS containing 0.05% Tween-20 and then treated with 0.1 mL/well of peroxidase-conjugated rabbit anti-goat IgG (1:2000 dilution in HBSS containing 1% skim milk). After 1-hour incubation at room temperature, the plates were washed 5 times with HBSS containing 0.05% Tween-20 and incubated at room temperature in 100 µL of 3% o-phenylene-diamine and 0.03% H₂O₂ in a mixture of 30 mmol/L citrate buffer and 100 mmol/L phosphate buffer, pH 7.4. After incubation for 15 minutes in a dark place, 50 µL/well of 2 mol/L H₂SO₄ was added, and spectrophotometric readings were made at 490 nm using a microplate reader. Because the cells were not permeabilized, this ELISA detected cell surface–expressed protein.

Western Blot Analysis

Western blot analysis was conducted to determine whether the changes in cell-surface expression of cell adhesion molecules by GBE depend on the changes in amounts of protein synthesis. Protein extracts were prepared as previously described.22 Briefly, HAECs were lysed in a buffer containing 62.5 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 0.5 mmol/L PMSF, 2 µg/mL aprotinin, 2 µg/mL pepstatin, and 2 µg/mL leupeptin. The whole-cell lysates were subjected to SDS-polyacrylamide (10%) gel electrophoresis, followed by electroblotting onto PVDF membrane. Membranes were probed with a mouse monoclonal antibody directed to NF-κB p65 (BD Transduction Laboratories) or goat antibodies directed to ICAM-1, VCAM-1, or E-selectin, incubated with horseradish peroxidase–labeled secondary antibody for 1 hour, and then washed with PBS containing 0.1% Tween 20. Bands were visualized by chemiluminescence detection reagents (NEN).

Electrophoresis Mobility Shift Assay

Transcriptional regulation involving activation of NF-κB and AP-1 has been implicated in the cytokine-induced expression of VCAM-1 and ICAM-1 genes. To examine whether GBE inhibits NF-κB or AP-1 activation, we performed electrophoresis mobility shift assay (EMSA) with the use of a 32P-labeled oligonucleotide with the consensus NF-κB and AP-1 binding sequence. Nuclear protein extracts were prepared as previously described.22 In brief, after being washed with ice-cold PBS, cells were scraped off the plates with a cell scraper in 1 mL of ice-cold buffer A (10 mmol/L HEPES/NaOH, pH 7.9; 10 mmol/L KCl; 1.5 mmol/L MgCl₂; 1 mmol/L DTT; 0.5 mmol/L PMSF; 0.1 mmol/L leupeptin; 0.5 mmol/L pepstatin; and 2 µg/mL leupeptin). After centrifugation at 300 g for 10 minutes at 4°C, cells were resuspended in 80 µL of buffer B (buffer A containing 0.1% Triton X-100) by gentle pipetting. Cell lysates were allowed to stand on ice for 10 minutes and then centrifuged at 12,000 g for 10 minutes at 4°C. Nuclear pellets were resuspended in 70 µL of ice-cold buffer C (20 mmol/L HEPES/NaOH, pH 7.9; 1.5 mmol/L MgCl₂; 1 mmol/L DTT; 0.2 mmol/L EDTA; 420 mmol/L NaCl; 25% glycerol; 0.5 mmol/L PMSF; 2 µg/mL aprotinin; 2 µg/mL pepstatin; and 2 µg/mL leupeptin), incubated on ice for 30 minutes with intermittent mixing, and then centrifuged at 15,000 g for 30 minutes at 4°C. Nuclear protein extracts prepared as described above were described above were determined by Bio-Rad protein assay (Bio-Rad Laboratories).

The probes for NF-κB and AP-1 in gel retardation assays were synthetic double-stranded oligonucleotides. For EMSA, the positive regulatory element was end-labeled with γ-3²P-adenosine-5′-triphosphate (ICN) using polynucleotide kinase (Promega) and purified by 2 passages through a Sephadex column (BM-Quick Spin columns DNA G25, Boehringer-Mannheim). The DNA binding reaction was conducted at room temperature for 20 minutes in a volume of 20 µL. Two micrograms of nuclear protein extracts were preincubated for 30 minutes on ice with 10 µg of salmon sperm DNA (Sigma-Aldrich). Protein and salmon sperm DNA were diluted in buffer D (20 mmol/L Na+(2-hydroxyethyl) piperazine-N′-2-(...
ethanesulfonic acid), pH 7.9, 20% glycerol, 0.1 mol/L potassium chloride, 0.2 mmol EDTA, 0.5 mmol phenylmethylsulfonyl fluoride, 0.5 mmol dithiothreitol, and 5 μg/mL leupeptin]. Protein extracts were diluted in buffer D to normalize the protein concentration in each lane. 32P-labeled positive regulatory element oligonucleotide 2 ng (200 000 to 500 000 cpm/ng) was added, and the reaction was allowed to proceed at room temperature for an additional 20 minutes. The samples were loaded onto 6% nondenaturing acrylamide gel (acrylamide/bisacrylamide 29:1) and run in ×0.25 Tris-Borate-EDTA buffer. Each gel was then vacuum dried and subjected to autoradiography.25 The specificity of the binding reaction was determined by coincubating duplicate samples with 100-fold molar excess of unlabeled oligonucleotide probe or anti-NF-κB (anti-p65) and anti-AP-1 (anti-c-Jun) antibodies.

Measurement of Reactive Oxygen Species Production
ROS has been shown to activate transcription factors in cultured endothelial cells and is implicated as a common second messenger in various pathways leading to NF-κB or AP-1 activation. The effect of GBE on ROS production in HAECs was determined by a fluorometric assay using 2,7’-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) as a probe for the presence of H2O2. Confluent HAECs (10⁵ cells/well) in 96-well plates were pretreated with various concentration of GBE for 18 hours. After the removal of GBE from wells, cells were incubated with 20 μmol/L DCFH-DA for 45 minutes. TNF-α was added to the medium for 60 minutes, and the fluorescence intensity (relative fluorescence units) was measured at 485-nm excitation and 530-nm emission using a fluorescence microplate reader.24

Statistical Analyses
Results were expressed as mean±SEM, and data were analyzed using one-way ANOVA followed by Dunn’s test or Student’s t-test for significant difference. Statistical significance was defined as P<0.05. All statistical procedures were performed with SigmaStat version 2.0 (Jandel).

Results

Ginkgo Biloba Extract Inhibits U937 Adherence to TNF-α–Activated Endothelial Cells
The numbers of U937 binding to HAECs in different conditions were shown by fluorescent microscopy in online Figure I (please see http://atvb.ahajournals.org). Unstimulated confluent HAECs exhibited minimal binding to HAECs. Endothelial adhesiveness to U937 was substantially increased when the HAECs were treated with TNF-α. Preincubation of confluent HAECs with GBE (0 to 100 μg/mL for 18 hours) inhibited U937 adhesion to HAECs treated with TNF-α (2 ng/mL for 6 hours) in a dose-dependent manner (Figure 1A). Preincubation of HAECs with probucol (a potent antioxidant as a positive control) similarly inhibited U937 adhesion to TNF-α–activated HAECs (Figure 1B). The maximal inhibition effect was achieved at 50 μg/mL in GBE (68.7±8.3% inhibition, P<0.05) and at 5 μg/mL in probucol (48.6±7.6% inhibition, P<0.05), respectively.

Additional study of the combination effects of probucol and GBE for monocyte/endothelial binding assay showed that there was no additive effect (Figure 1C), suggesting that GBE and probucol may share a common mechanism, such as antioxidant effect, to inhibit TNF-α–activated endothelial adhesiveness.

Ginkgo Biloba Extract Inhibits Total Protein Expression of Cell Adhesion Molecules
Treatment of HAECs with TNF-α (2 ng/mL) for 6 hours significantly increased the amounts of total protein for VCAM-1 and ICAM-1 on Western blot analysis. Pretreatment with GBE (50 μg/mL) markedly suppressed TNF-α–induced total protein expression of VCAM-1 and ICAM-1 (37% and 31% inhibition, respectively; P<0.05). Pretreatment with probucol (5 μmol/L) had similar effect in suppressing TNF-α–induced VCAM-1 protein expression (55% inhibition, P<0.05) but did not affect ICAM-1 protein expression. The expression of E-selectin was not altered either by GBE or by probucol (Figure 2).

Ginkgo Biloba Extract Inhibits NF-κB and AP-1 Activation of NF-κB and AP-1
The results of EMSA showed that treatment with TNF-α (2 ng/mL for 30 minutes at 37°C) resulted in the appearance of both NF-κB (Figure 3A) and AP-1 (Figure 3B) shifted bands. These bands were specific for NF-κB or AP-1 binding, because they were undetectable when a 100-fold excess of unlabeled NF-κB or AP-1 oligonucleotide was included (Figure 3C). Pretreatment with either GBE (50 μg/mL) or probucol (5 μmol/L) significantly reduced the densities of NF-κB shifted bands induced by TNF-α (55.2% and 65.6% inhibition, respectively; P<0.05) (Figure 3A). However, only GBE could significantly reduce the AP-1 shifted bands induced by TNF-α (45.1% inhibition, P<0.05). The effect of probucol on AP-1 was much less (22.3% inhibition, P=NS) (Figure 3B).
Ginkgo Biloba Extract Inhibits TNF-α–Induced Intracellular ROS Production

Figure 4 showed the effects of GBE (50 μg/mL) and probucol (5 μmol/L) on intracellular ROS production induced by TNF-α (2 ng/mL for 1 hour) in HAECs. Pretreatment with GBE (from 0, 6.25, 12.5, 25, 50, or 100 μg/mL) or probucol (0, 1.25, 2.5, 5, 10, or 20 μmol/L) or both followed by TNF-α (2 ng/mL for 6 hours). Dose-dependent effects of anti-VCAM-1 antibody on TNF-α–stimulated adhesiveness of HAECs to U937 monocytic cells. HAECs were pretreated for 18 hours with anti-VCAM-1 antibody (1, 2, 5, or 10 μg/mL) followed by TNF-α (2 ng/mL for 6 hours). Binding between U937 and TNF-α–stimulated HAECs was measured as described in Methods. The results for 3 separate experiments, each performed in triplicate, are expressed as mean number of adherence cells per high-power field ± SEM. *P<0.05 compared with TNF-α–stimulated HAECs or with anti-VCAM-1 antibody 1 μg/mL.

Discussion

The cardinal findings of this study indicated that in cultured HAECs, pretreatment with GBE, a complex mixture with the main active ingredients flavonoids and terpene lactones, significantly suppressed TNF-α–induced intracellular ROS formation, the activation of redox-sensitive transcription factors NF-κB and AP-1, the expression of VCAM-1 and ICAM-1, and the adhesiveness to a human monocytic cell line (U937), an in vitro sign of atherogenesis. Because TNF-α could stimulate intracellular ROS production and activate redox-sensitive transcription pathways that contribute to endothelial adhesion molecule expression, it is then suggested that GBE could directly inhibit vascular inflammation and prevent in vitro atherogenesis by modulating endothelial redox-sensitive transcription pathways such as NF-κB and AP-1.

Previous studies have shown that GBE could directly scavenge superoxide anion, hydroxyl radicals, peroxyl radical species, and nitric oxide. It was also shown to reduce lipid peroxidation and LDH release and elevate intracellular GSH level and GSSG reductase activity in endothelial cells. Thus, the inhibitory effect of GBE on TNF-α–induced activation of NF-κB and AP-1 could be theoretically attributable to its antioxidant properties that directly scavenging...
intracellular H₂O₂ or elevating GSH levels or both. Besides, it was shown that combination of GBE and probucol, an antioxidant, had no additional inhibition effect on TNF-α-activated endothelial adhesiveness, supporting the notion that GBE and probucol may share a common mechanism to inhibit intracellular redox-sensitive transcription pathways.

In the present study, GBE inhibited the activation of both NF-κB and AP-1 and the expression of both VCAM-1 and ICAM-1, whereas probucol only inhibited NF-κB activation and VCAM-1 expression in HAECs. The expression of E-selectin was unaffected by GBE or probucol. The results agree in part with those of some previous studies. It was shown that in human umbilical vein endothelial cells, pyrrolidine dithiocarbamate, an inhibitor of NF-κB activation, markedly attenuates TNF-α-induced surface protein and mRNA expression of VCAM-1 but not that of ICAM-1 or E-selectin. It was also reported that pretreatment with probucol significantly reduced oxidized LDL-induced VCAM-1 expression in human umbilical vein endothelial cells. Taken together, NF-κB may regulate VCAM-1 expression and should frequently function together with other transcription factor such as AP-1 to regulate the expression of other adhesion molecules. In fact, AP-1-mediated induction of ICAM-1 could occur independently of the activation of NF-κB pathway. Our findings are then compatible with the current understanding that endothelial expression of VCAM-1 is mainly mediated by the activation of NF-κB whereas ICAM-1 expression is mediated by the activation of both NF-κB and AP-1. Besides, because pretreatment with GBE and probucol could inhibit monocytes’ adhesiveness to endothelial cells in a similar context, it seems that endothelial VCAM-1 rather than ICAM-1 expression is more critical to monocyte adhesion in this in vitro model.

In the present study, the inhibitory effects of GBE and probucol on intracellular ROS production could be only partially translated to the inhibition of NF-κB activation and its downstream adhesion molecule expression in TNF-α-stimulated HAECs. These findings are also compatible with the present understanding that the activation of NF-κB by cytokines such as TNF-α could be caused through both redox-dependent and -independent pathways. For example, simultaneously applied H₂O₂ could strongly potentiate the TNF-α-induced transcriptional activity of NF-κB, suggesting the possible presence of an oxidative stress-independent mechanism induced by TNF-α. Besides, thioredoxin, a 12-kDa endogenous protein that modulates the redox state of proteins, could only selectively inhibit TNF receptor-associated factor-mediated but not mitogen-activated protein kinase-mediated NF-κB activation, suggesting complex mechanisms, including both oxidative stress-dependent and oxidative stress-independent pathways of TNF-α-induced NF-κB activation.

The dose of GBE used in previous in vitro studies usually ranged from 200 to 400 μg/mL. In the present study, a dose of 50 μg/mL GBE contained approximately 9 μmol/L quercetin glycoside and 20 μmol/L total flavonoid glycoside. Commercial purveyors of Ginkgo biloba tablets recommend a daily dose of 120 to 240 mg. Thus, a single dose of commercial Ginkgo biloba would not reach the levels of extract used in the present studies. However,
the pharmacokinetics of the components of *Ginkgo biloba* have not been completely understood. It is possible that accumulation of some of the components may occur with prolonged treatment and may also concentrate in different tissues.39,40

In conclusion, GBE could prevent in vitro atherogenesis, probably via its antioxidant properties by modulating redox-sensitive transcription pathways and reducing endothelial adhesion molecule expression. The findings may provide a rationale for the in vivo antiatherosclerosis effect of GBE41.

**Figure 3.** Inhibitory effects of GBE or probucol on TNF-α-induced NF-κB (A) or AP-1 (B) activation in HAECs. Confluent HAECs were preincubated for 18 hours either with 50 μg/mL GBE or 5 μmol/L probucol followed by 30-minute incubation with 2 ng/mL TNF-α. Nuclear protein extracts were prepared, and a gel shift assay was performed using radiolabeled oligonucleotides containing consensus NF-κB or AP-1 binding sequences, as described in Methods. The results are from 3 separate experiments. *P<0.05 compared with TNF-α effect. To confirm that the presence of bands is specific to NF-κB or AP-1, unlabelled oligonucleotide controls were also done in the separated studies (C).
and support its potential use in clinical atherosclerosis disease.

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Figure 1. Effects of GBE or probucol on TNF-α-stimulated adhesiveness of HAECs to U937 monocytic cells. HAECs were pretreated for 18 hours with GBE (50 µg/ml) or probucol (5 µM), followed by TNF-α (2 ng/ml for 6 hours). Representative fluorescence photomicrographs showing effects of drug treatment on the TNF-α-induced adhesion of DCF-labeled U937 monocytic cells to HAECs. (A): Control; (B): TNF-α treatment; (C): GBE+TNF-α treatment; (D): Probucol+TNF-α treatment. Three independent experiments gave similar results.