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Inhibitory Effect of *Ginkgo biloba* Extract on the Expression of Inducible Nitric Oxide Synthase in Endothelial Cells

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ABSTRACT. Excessive production of nitric oxide (NO) may have cytotoxic effects through the formation of peroxynitrite with superoxide. The extract of *Ginkgo biloba* leaves (EGb) has been demonstrated to be a potent scavenger of free radicals. Although EGb has been shown recently to inhibit NO production in macrophages, its effect on NO production in endothelial cells is largely unknown. The objective of this study was to elucidate the mechanism by which EGb affects NO production in a human endothelial cell line (ECV304). After cells were incubated with EGb (10–100 μg/mL) for 2 or 4 hr, the amounts of NO metabolites released by the cells were quantitated, and cellular NOS activities were determined following the conversion of [^{3}H]arginine to [^{3}H]citrulline. NOS protein expression was determined by western immunoblotting analysis. mRNA levels were examined by reverse transcription–polymerase chain reaction (RT–PCR) analysis. EGb (50 μg/mL) caused a 30% reduction of NO metabolites released by endothelial cells. Following EGb treatment, cellular inducible NO synthase (iNOS) activity was reduced by 28% with a concomitant reduction in the levels of iNOS protein mass and mRNA. There was no change in the activity or protein mass of constitutive NO synthase in these cells. EGb inhibited NO production by attenuating the level of iNOS mRNA in ECV304 cells. Selective inhibition of iNOS by EGb may be therapeutically relevant in modulating NO production in endothelial cells. BIOCHEM PHARMACOL **58**;10:1665–1673, 1999. © 1999 Elsevier Science Inc.

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Vascular endothelial cells produce various cytokines, growth factors, and active molecules that regulate physiological and pathological processes. Endothelial cell-derived NO§ is an important mediator in the cardiovascular system as well as in the nervous and immune systems [1–3]. NO is synthesized from L-arginine in endothelial cells by a reaction catalyzed by the enzyme NOS [1–3]. Several isoforms of NOS have been identified, namely cNOS, which is calcium/calmodulin dependent, and iNOS, which is not regulated by changes in concentrations of calcium/calmodulin [4–6]. The constitutive form of the enzyme is the predominant isoform expressed in endothelial cells [5, 7, 8], thus leading to the use of the term eNOS (endothelial enzyme) interchangeably with cNOS. cNOS is also present in neuronal cells [9]. iNOS, on the other hand, is expressed mainly in macrophages [10, 11] and has been found in other cells, including endothelial cells [12], cardiac myocytes [13], vascular smooth muscle cells [14], hepatocytes [15], and mesangial cells [16]. Under normal conditions, endothelial cNOS-derived NO acts as a vasodilator that plays an important role in the regulation of vessel tone [1–3, 5]. Therefore, the physiological concentration of cNOS-derived NO is under strict regulation, primarily by calcium via the calcium-binding protein calmodulin [4–6]. For example, increased influx of calcium into endothelial cells can cause an activation of cNOS, leading to an increase in NO levels.

NO is also a principal mediator in various pathological processes [5]. Unbalanced production (over- or underproduction) of NO has been observed in atherosclerosis and other injuries causing endothelial dysfunction [5, 17, 18]. Excessive production of NO by iNOS may have cytotoxic effects through the formation of peroxynitrite with superoxide [5, 17, 18]. Unlike cNOS, which is sensitive to Ca^{2+}, iNOS is regulated mainly through the induction of *de novo* enzyme synthesis [6, 19, 20]. iNOS in various cells can be induced by cytokines, growth factors, and endotoxins (i.e. lipopolysaccharide) at the levels of gene transcription and translation [6]. Once iNOS is induced, NO production at high levels will be sustained for a prolonged period of time. Excessive production of NO by iNOS can cause endothelial damage leading to multiple injuries in vessel walls [6, 17, 18]. For example, macrophage-derived NO may contribute

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§ *Abbreviations*: NO, nitric oxide; EGb, extract(s) of *Ginkgo biloba* leaves; NOS, nitric oxide synthase; RT–PCR, reverse transcription–polymerase chain reaction; L-NAME, N^G-nitro-L-arginine-methyl ester; iNOS, inducible nitric oxide synthase; and cNOS, constitutive nitric oxide synthase.

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to the development of atherosclerotic lesions through oxidation of lipoproteins within vessel walls [18]. iNOS also has been suggested to augment injury elicited by oxidative stress in cardiac myocytes [14]. However, the contribution of endothelial cell-derived iNOS to the excessive production of NO within vessel walls remains to be determined.

Factors that can selectively regulate the activities of various NOS isoforms may have therapeutic implications in the treatment of cardiovascular disorders. Many efforts have been made to identify inhibitors that can inhibit iNOS selectively while leaving cNOS untouched [21–24]. Extracts from *Ginkgo biloba* tree leaves (EGb) have been used therapeutically as a traditional herbal medicine. Recently, a defined extract prepared from *Ginkgo biloba* leaves has been marketed for the treatment of cardiovascular or cerebrovascular disorders [25, 26]. Several mechanisms have been proposed for the observed effects of *Ginkgo biloba* extracts [27–31]. For example, the extract may act directly as a NO scavenger as well as inhibit NO production in activated macrophages [30], or it may act via its antioxidant effect [27–29]. However, its effect on endothelial NO production is largely unknown.

In the present study, the effect of EGb on NO production in endothelial cells was investigated. Our results demonstrated that human endothelial cells (ECV304), which are spontaneously transformed umbilical vein endothelial cells, were able to produce detectable amounts of NO under defined culture conditions [32–34]. These cells expressed both calcium-dependent and calcium-independent forms of NOS activities (cNOS and iNOS). EGb, at a concentration of 50 μg/mL, was shown to reduce the production of NO significantly in ECV304 cells. Further analysis revealed that the decrease in NO production was accompanied by a reduction in the expression of iNOS mRNA in EGb-treated cells.

MATERIALS AND METHODS

Culture of Endothelial Cells

The human endothelial cell line ECV304 was purchased from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (penicillin, 100 U/mL; streptomycin, 10 μg/mL; and neomycin, 20 μg/mL). For experiments, cells were plated in 35-mm dishes and cultured in 1 mL of RPMI-1640 medium in the absence or presence of EGb. The EGb used in this study was obtained from the Shanghai Luyuan Industry Company Ltd. It is a standardized extract (EGb) that contains 24% flavonoid glycosides and 6% terpenoids [35, 36]. This EGb is compatible with the extracts prepared by other laboratories, i.e. IPSEN Institute, France [25, 30]. Prior to experiments, EGb was dissolved in 5% ethanol (10 mg/mL). The final concentrations of ethanol (0.01 to 0.05%) present in the culture medium did not affect the release of NO metabolites or the NOS activities in endothelial cells.

Assay of Nitrite and Nitrate

Cells were cultured without or with EGb (10–100 μg/mL) for various periods of time. At the end of the experimental period, medium was collected, and the amount of NO released by cells was determined by using an NO assay kit (Calbiochem) according to the manufacturer's protocol. Briefly, the method involved measuring the amount of NO metabolites (nitrite and nitrate), which were more stable than NO. Since the Griess reagent reacts only with nitrite, nitrate in the culture medium was reduced first to nitrite by the action of nitrate reductase. Then the reaction was initiated by the addition of Griess reagent, which contained 1% (w/v) sulfanilamide and 0.1% (w/v) *N*-[1-naphthyl]-ethylenediamine. The absorbance of samples was read at 540 nm.

Determination of NOS Activity

NOS activities associated with cells were determined following the conversion of [^{3}H]arginine to [^{3}H]citrulline [37, 38]. Cells were lysed in 30 mmol/L of HEPES buffer (pH 7.4) containing 1 μmol/L of leupeptin, 0.1% Triton X-100, and 1 μmol/L of phenylmethylsulfonyl fluoride. An aliquot of cell lysate was used for the determination of NOS activities. The reaction mixture contained 30 mmol/L of HEPES buffer (pH 7.4), 1 mmol/L of $CaCl_2$, 100 μmol/L of NADPH, 300 μmol/L of tetrahydrobiopterin, 1 mmol/L of dithiothreitol, and 40 μmol/L of L-[^{3}H]arginine (37 kBq/reaction mixture) as substrate. For determination of calcium-independent NOS activity, $CaCl_2$ was omitted and 1 mmol/L of EGTA was added. The reaction was carried out at 37° for 60 min. The reaction was terminated by the addition of 200 μL of stop buffer containing 100 mmol/L of HEPES (pH 5.5) and 10 mmol/L of EGTA. Separation of [^{3}H]arginine and the reaction product [^{3}H]citrulline in the mixture was achieved using a 5-mL column of Dowex ion-exchange resin. Radioactivity associated with citrulline was determined by liquid scintillation counting.

Western Immunoblotting Analysis

Equal amounts of cellular proteins from control or EGb-treated cells were separated on an SDS–7.5% polyacrylamide gel followed by electrophoretic transfer of proteins from the gel to nitrocellulose membrane (pore size, 0.45 μm) [39]. The membrane was probed with either rabbit anti-NOS antibodies (Calbiochem), which recognized 140-kDa human endothelial NOS (cNOS), or rabbit anti-inducible NOS antibodies (Calbiochem), which recognized 130-kDa human iNOS. Blots were developed using horseradish peroxidase-conjugated secondary antibodies. Bands corresponding to NOS proteins, identified by similarity in relative mobility on the SDS–polyacrylamide gel to positive controls supplied by the manufacturer, were visualized using enhanced chemiluminescence reagents (Amersham) and

analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst® version 1.1).

Analysis of NOS mRNA

Total RNA from cultured cells was isolated with TRIzol Reagent (Life Technologies). RT–PCR was performed by using a RT–PCR Amplimer Set for iNOS purchased from Clontech Laboratories Inc. The primers used in the PCR reactions (5′-CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3′; and 5′-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3′) were synthesized by the same company. The reaction mixture for PCR contained 1.5 mmol/L of $MgCl_2$, 0.2 mmol/L of dNTP, 0.4 μmol/L of 5′ primer, 0.4 μmol/L of 3′ primer, 2 units *Taq* DNA polymerase, and 1 μg of cDNA product from the reverse transcription reaction. After an initial denaturation for 2 min at 95°, 30 cycles of PCR amplification (95° for 45 sec, 60° for 45 sec, and 72° for 2 min) were carried out, followed by an additional 7-min extension at 72°. The cDNA amplification was linear for up to 30 cycles of PCR. The products of PCR were separated by electrophoresis on 1.5% agarose gel (containing ethidium bromide) and visualized under UV using a gel documentation system (Bio-Rad Gel Doc1000). Human β-actin was used as an internal standard to verify equal PCR product loading for each experiment. After RT–PCR, the NOS signal was normalized by comparing with the β-actin signal from the same sample [40]. The values are expressed as a ratio of iNOS to β-actin. In negative controls, reverse transcriptase was omitted during the reverse transcription reaction.

Cell Proliferation

The incorporation of [^{3}H]thymidine into cellular DNA was measured to examine the effect of EGb on cell proliferation. ECV304 cells were cultured in RPMI-1640 medium in the absence or presence of EGb for 4 hr. After incubation, cells were washed with PBS containing 1% BSA and then lysed in 0.2 mol/L of sodium hydroxide. The radioactivity associated with cellular DNA was determined by liquid scintillation counting [41]. Cell proliferation also was examined by counting cell numbers under a light microscope.

Statistical Analysis

Student's *t*-test was used for statistical analysis between two groups. The level of statistical significance was set at $P < 0.05$.

RESULTS

Effect of EGb on NO Release by Endothelial Cells

The effect of EGb on NO release by ECV304 cells was examined first. Cells were incubated in RPMI-1640 containing EGb (50 μg/mL) for 2 and 4 hr. The amounts of NO metabolites (nitrate and nitrite) released into the culture medium were measured by the Griess reaction method. As shown in Fig. 1, pretreatment of cells with EGb (50 μg/mL) for 2 hr did not have a significant effect on the amount of NO metabolites released by ECV304 cells. After cells were incubated with EGb for 4 hr, the amount of NO metabolites released into the culture medium was reduced significantly (to 70% of control). Incubation of cells with EGb for 12 hr did not result in further inhibition of the release of NO metabolites from those cells. The amount of NO metabolites released into the culture medium appeared to reach a plateau after 4 hr of incubation (data not shown). Cells then were incubated with various concentrations of EGb (10–100 μg/mL) for 4 hr, and the amount of NO released into the culture medium was determined. As shown in Fig. 2, EGb inhibited the release of NO metabolites by endothelial cells in a concentration-dependent manner. To determine if this inhibitory effect was calcium dependent, cells were incubated with EGb in the absence or

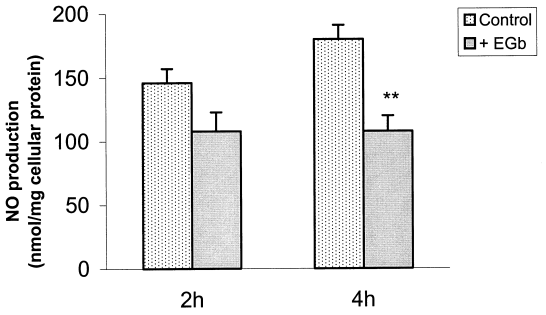

FIG. 1. NO production in endothelial cells. Cells were incubated in RPMI-1640 containing EGb (50 μg/mL) for 2 and 4 hr. At the end of the incubation period, culture medium was collected, and aliquots were used to quantitate the level of NO (nitrate and nitrite) as described in Materials and Methods. Results are expressed as means ± SD for five separate sets of experiments. In control experiments, cells were cultured in RPMI-1640 in the absence of EGb for 2 and 4 hr. Key: (**) $P < 0.01$ when compared with the control value.

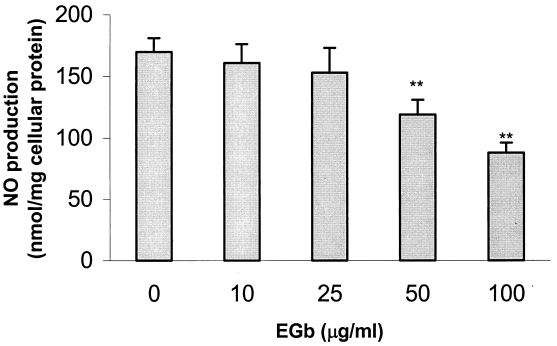

FIG. 2. Effect of EGb at various concentrations on NO production in endothelial cells. Cells were incubated in RPMI-1640 containing various amounts of EGb for 4 hr. After the incubation period, culture medium was collected, and aliquots were used for determination of NO (nitrate and nitrite) levels. Results are expressed as means ± SD for five separate sets of experiments. In control experiments, cells were cultured in the absence of EGb. Key: (**) $P < 0.01$ when compared with the control value.

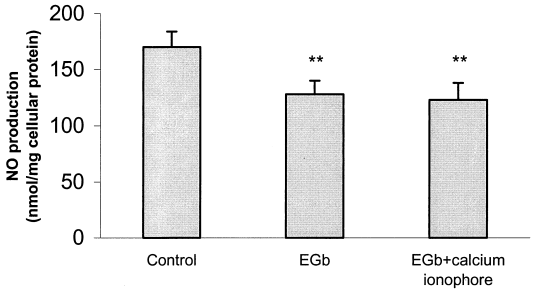

FIG. 3. Effect of a calcium ionophore on NO production in EGb-treated endothelial cells. Cells were incubated in RPMI-1640 for 4 hr in the presence of EGb (50 μg/mL) or EGb plus the calcium ionophore A23187 (0.2 nM) for 4 hr. After incubation, culture medium was collected, and aliquots were used for determination of NO (nitrate and nitrite) levels. Results are expressed as means ± SD from three separate sets of experiments. In control experiments, cells were cultured in the absence of EGb and the calcium ionophore. Key: (**) $P < 0.01$ when compared with the control value.

presence of the calcium ionophore A23187 (Sigma). As shown in Fig. 3, inclusion of the calcium ionophore in the culture medium did not change the inhibitory effect of EGb on NO release from endothelial cells. These results suggested that the effect of EGb on endothelial cells was not dependent on changes in intracellular calcium concentration.

Effect of EGb on NOS Activity in Endothelial Cells

Decreased NO release in EGb-treated cells indicated that the synthesis of NO might be altered in these cells. Therefore, the NOS activities were measured in cultured ECV304 cells. After incubation with EGb for 4 hr, cellular NOS activities were determined. First, the assay for NOS activity was conducted in the presence of calcium. As depicted in Fig. 4A, total NOS activities (the sum of calcium-dependent and calcium-independent activities) were inhibited significantly after incubation of cells with 50 or 100 μg/mL of EGb. Second, to test whether this inhibitory effect was calcium dependent, the assay for NOS activity was carried out in the absence of calcium. As shown in Fig. 4B, calcium-independent NOS activity was inhibited significantly after treatment of cells with EGb. The cNOS activity was calculated based on the difference between the total NOS activity and the iNOS activity. As shown in Fig. 4C, cNOS activity was not affected significantly in EGb-treated cells. These results indicated that the inhibitory effect of EGb on NOS activity in ECV304 cells was calcium independent, while the calcium-dependent NOS activity was relatively unchanged. Finally, the direct effect of EGb on NOS activity in ECV304 cells was determined *in vitro*. As depicted in Fig. 5, the addition of EGb (25–100 μg/mL) to the assay mixture did not have any effect on the activity of NOS. When an analogue of L-arginine, L-NAME, which is an inhibitor of all the isoforms of NOS, was added to the assay mixture, the NOS

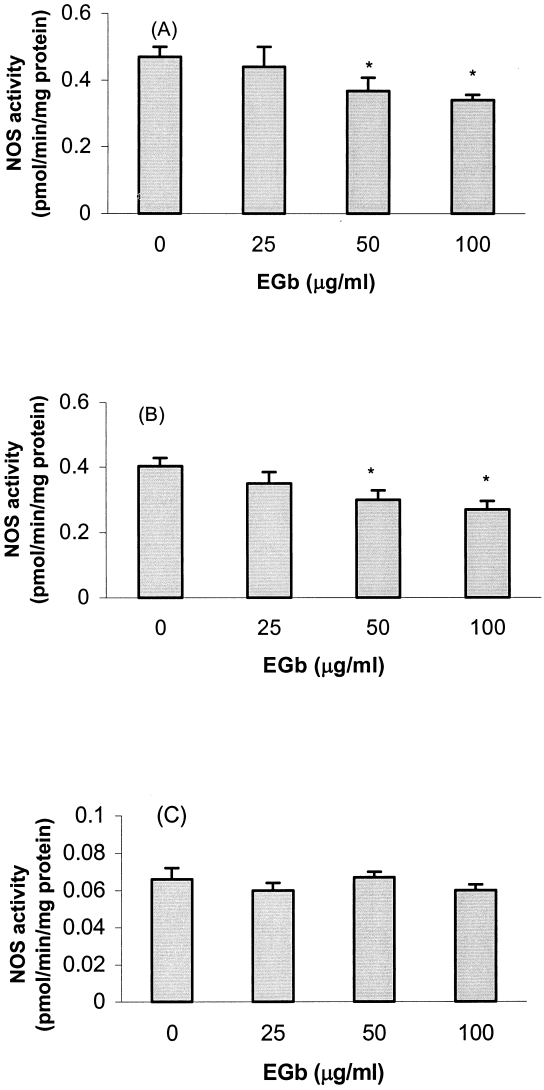

FIG. 4. Effect of EGb on NOS activity in endothelial cells. Cells were incubated in RPMI-1640 containing various amounts of EGb for 4 hr. At the end of the incubation period, culture medium was removed, and cellular NOS activity was determined as described in Materials and Methods. The results are depicted as means ± SD for three separate sets of experiments. In control experiments, cells were cultured in RPMI-1640 in the absence of EGb for 4 hr. (A) Total NOS activity. The assay for NOS activity was conducted in the presence of calcium. (B) iNOS activity. The assay for NOS activity was conducted in the absence of calcium. (C) cNOS activity. The difference between total NOS activity and iNOS activity was calculated by subtracting the iNOS activity from the total NOS activity. Results are depicted as means ± SD for three separate sets of experiments. Key: (*) $P < 0.05$ when compared with the control values.

activity was decreased markedly (Fig. 5). Taken together, these results suggested that the decreased NO production in EGb-treated cells was accompanied by a reduction in the iNOS activity. The inhibitory effect of EGb on cellular iNOS activity was not due to a direct action of EGb on the NOS enzyme. Lipopolysaccharide treatment has been shown to induce the expression of iNOS in many types of

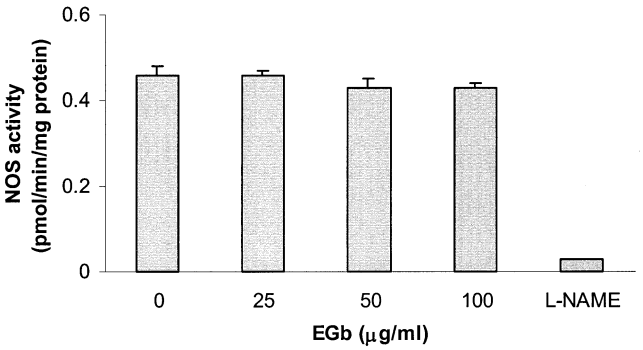

FIG. 5. Direct effect of EGb on NOS activity. Aliquots of cell lysate were used for the determination of NOS activity in the absence or presence of EGb (25–100 μg/mL) or L-NAME (50 mmol/L). Results are expressed as means ± SD for three separate experiments.

cells [6, 21, 30]. The inducibility of iNOS in ECV304 cells also was examined. Preincubation of cells with lipopolysaccharide (0.01 to 10 μg/mL) for 2–24 hr did not result in significant changes in the amount of NO metabolites released from cells or in the activities of cNOS and iNOS. The iNOS in ECV304 cells appeared to be up-regulated prior to any induction.

Effect of EGb on the Expression of NOS Proteins in Endothelial Cells

Decreased NOS activities in EGb-treated cells might be due to a reduction in the levels of enzyme proteins. Therefore, the effect of EGb on the expression of NOS proteins in endothelial cells was examined by western immunoblotting analysis. Immunoblotting using polyclonal anti-cNOS or anti-iNOS antibodies identified two bands with estimated molecular masses of 140 kDa (cNOS) and 130 kDa (iNOS), respectively. As shown in Fig. 6, 50 and 100 μg/mL of EGb treatment caused a 27 and 43% reduction in the levels of iNOS protein, respectively. On the other hand, there was no significant change in the levels of cNOS protein in the same samples (Fig. 6).

Effect of EGb on the Expression of NOS mRNA in Endothelial Cells

A reduction in iNOS protein levels in EGb-treated cells might be a result of changes in the expression of iNOS mRNA in these cells. To determine whether EGb affected the expression of iNOS mRNA, cells were preincubated with EGb (50 or 100 μg/mL) for 4 hr, and total cellular RNA was extracted. The effect of EGb on the expression of iNOS mRNA was examined by an RT–PCR method. As shown in Fig. 7, EGb inhibited the expression of iNOS mRNA in a concentration-dependent manner. After cells were treated with EGb at concentrations of 50 and 100 μg/mL, the ratio of iNOS to β-actin was reduced from 0.22 (control) to 0.16 and 0.14, respectively, reflecting a significant decrease in levels of mRNA as compared with the control. These results indicated that decreased production of NO in endothelial cells was a result of reduced levels of iNOS mRNA in EGb-treated cells. A time–course experiment on iNOS expression in ECV304 cells in the absence and presence of EGb also was performed. Cells were incubated in the absence or presence of EGb (50 μg/mL) for various time periods; total cellular RNA was extracted, and RT–PCR was performed. As shown in Fig. 8, the levels of iNOS mRNA decreased to 60–70% in cells treated with EGb for 2, 4, 8, and 12 hr. Although the data obtained did not reflect the absolute change in the levels of iNOS mRNA, the relative change in iNOS mRNA levels in EGb-treated cells compared with those in control cells was noted.

Effect of EGb on DNA Synthesis in Endothelial Cells

To determine whether EGb had any effect on cell proliferation, total DNA synthesis was estimated by measuring the incorporation of [^{3}H]thymidine into cellular DNA. As shown in Fig. 9, EGb at concentrations of 50 and 100 μg/mL had no effect on the incorporation of radioactivity into endothelial cells. Endothelial cell proliferation also was determined by counting the total cell number after incubation with EGb. There was no change in cell number as compared with the control (data not shown). These results suggested that EGb at the concentrations tested did not affect DNA synthesis.

DISCUSSION

Endothelial cell-derived NO is involved in various physiological as well as pathological processes [1–5, 17, 18]. Excessive production of NO by endothelial cells may have a cytotoxic effect on vascular walls [17, 18]. In the present study, EGb was shown to attenuate the production of NO in endothelial cells. This inhibitory effect was a result of decreased expression of iNOS mRNA upon EGb treatment.

The NO molecule plays a dual role in the development of vascular disorders such as atherosclerosis [5]. At physiological concentration (basal levels), cNOS-derived NO can cause vasorelaxation, decrease platelet aggregation, prevent monocyte adhesion to the vessel wall, and suppress lipoprotein oxidation [1, 3–5, 37, 38]. On the other hand, excessive production of NO, especially by the action of iNOS, may be cytotoxic to the vessel wall by imposing oxidative stress as well as regulating gene expression of other molecules [5, 6, 23]. Macrophage iNOS has been thought to be responsible for the overproduction of NO in injured tissues [21]. However, during the pathologic process, endothelial iNOS also might be up-regulated and, hence, could contribute to the excessive production of NO in the local tissues. Human endothelial cells (ECV304) purchased from the American Type Culture Collection are spontaneously transformed umbilical cord vein endothelial cells. These cells have been used by a number of investigators to study endothelial functions [32–34]. However,

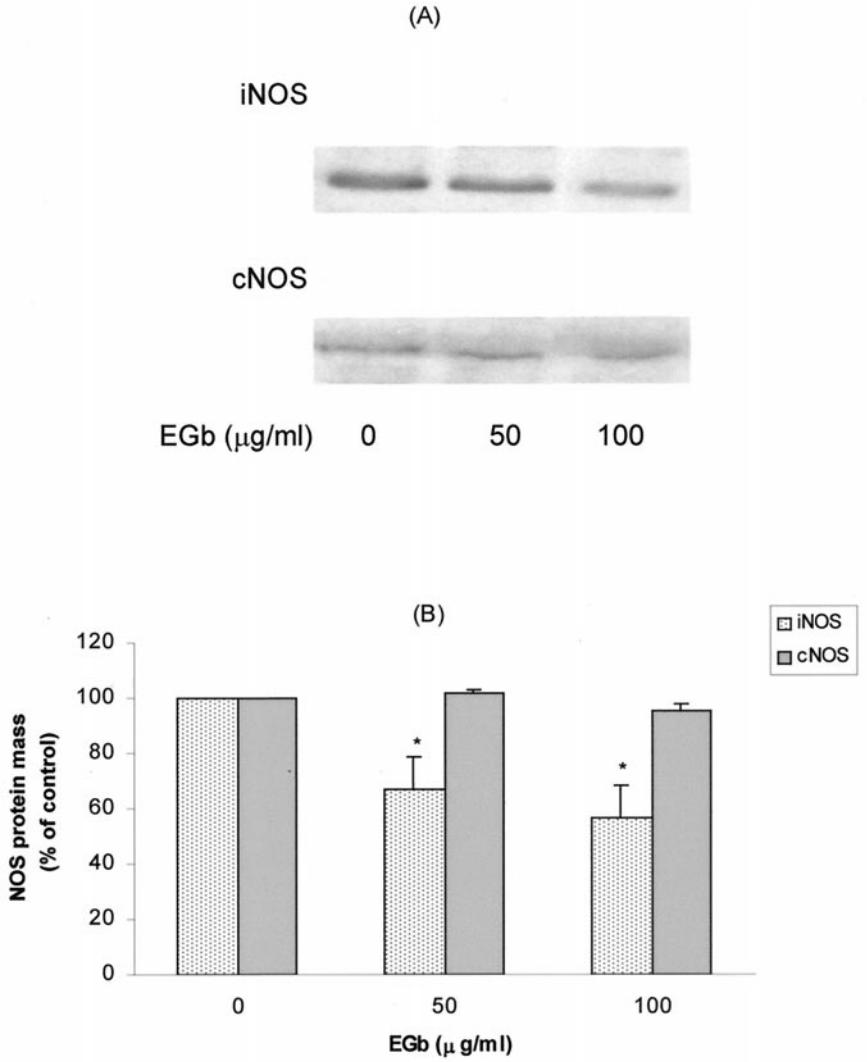

FIG. 6. Western immunoblotting analysis of NOS proteins in endothelial cells. Cells were preincubated in the absence or presence of EGb for 4 hr. Equal amounts of cellular proteins from control or EGb-treated cells were separated on an SDS–7.5% polyacrylamide gel, followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. (A) The bands of NOS were identified by western immunoblotting with anti-NOS antibodies. (B) The immunoblots were analyzed by densitometry, and the data were generated as integrated density units. Results are expressed as a percentage of control and depicted as means ± SD for three separate sets of experiments. In control experiments, cells were cultured in the absence of EGb. Key: (*) $P < 0.05$ when compared with the control values (0.83 ± 0.11 relative densitometric units for iNOS and 1.25 ± 0.22 relative densitometric units for cNOS).

little information has been available on NO production in these cells. In the present study, these cells exhibited both calcium-dependent and calcium-independent NOS activities that were sensitive to L-NAME, an inhibitor of NOS. These observations allowed the utilization of this cell line as a facile model system to study the regulation of NO production, NOS activities, and the protein and mRNA expression of both cNOS and iNOS.

Macrophage iNOS is expressed in response to various stimuli, including cytokines and endotoxins, in immune responses as well as in various pathologic processes [17–20]. The induction of iNOS expression in human cells requires stimulation by a combination of several cytokines and usually requires a lag period of 4–8 hr [21]. Although the expression of macrophage iNOS is regulated mainly at the transcriptional and translational levels [6, 19, 20], iNOS expression can also be interfered with at the post-translational level [42]. For example, transforming growth factor β has been shown to regulate macrophage iNOS expression through a combination of decreased iNOS mRNA stability and increased iNOS protein degradation [43]. On the other hand, oxidized LDL has been shown to have a direct inhibitory effect on iNOS enzyme activity in the mouse monocyte/macrophage cell line J774.A1 [42]. In the present study, EGb was clearly demonstrated to attenuate NO production in endothelial cells. This inhibitory effect was not a result of a direct interaction between EGb and the NOS protein. The inhibitory effect of EGb on NO production appeared to correlate with a reduction in iNOS protein levels as well as in mRNA levels in these cells. Interestingly, EGb did not seem to affect the expression of cNOS in these cells. EGb has been shown to reduce ligand

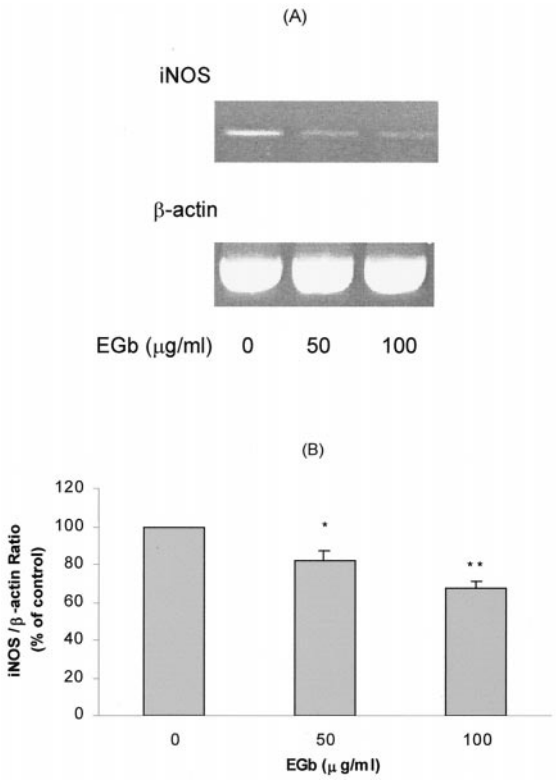

FIG. 7. Effect of EGb on the expression of iNOS mRNA in endothelial cells. The expression of mRNA for iNOS was determined by RT–PCR analysis. (A) 1.5% agarose gel electrophoresis of PCR-amplified cDNA derived from iNOS and human β-actin stained with ethidium bromide. (B) Quantitation of mRNA levels by densitometry. The levels of iNOS mRNA and β-actin mRNA were quantified by densitometry. The iNOS expression was normalized by expressing the data as the ratio of iNOS to β-actin. Results are expressed as means ± SD for three separate sets of experiments. Key: (*) $P < 0.05$ or (**) $P < 0.01$ when compared with the control value.

binding as well as protein and mRNA expression of the adrenal mitochondrial peripheral benzodiazepine receptor [44]. EGb at the concentrations tested in this study did not affect the proliferation of ECV304 cells. It remains to be determined whether the attenuation of iNOS mRNA levels by EGb is a result of a decrease in mRNA stability, an increase in mRNA degradation, or a decrease in transcription.

EGb has been used to treat vascular disorders for many years in several countries [25, 26]. Recently, clinical efficacy of the use of EGb in the treatment of dementia of the Alzheimer type and multi-infarct dementia has been demonstrated in several studies [45–48]. Elevated cerebral vascular production of NO and NOS activities also has been shown in Alzheimer's disease-derived microvessels [49]. It has been shown that one of the neuroprotective mechanisms of EGb might be through the reduction of NO production via the inhibition of NOS activity [49]. A few studies have been conducted to investigate the chemical composition and underlying mechanisms of its therapeutic effects on cardiovascular or cerebrovascular disorders [27–

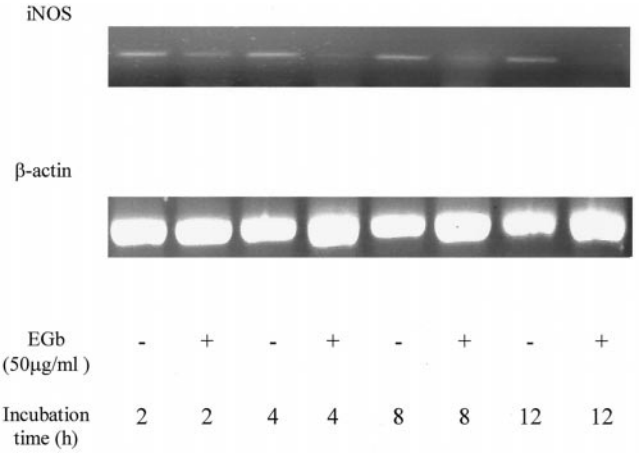

FIG. 8. Time course of the expression of iNOS mRNA in endothelial cells. Cells were incubated with EGb (50 μg/mL) for 2–12 hr. After incubation, total RNA was isolated, and RT–PCR analysis was performed with iNOS and β-actin. PCR-amplified cDNAs derived from iNOS or β-actin were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

31]. One of the mechanisms was thought to be direct scavenging of NO [27–30]. It has been shown that EGb (20–200 μg/mL) has an inhibitory effect on NO production in the macrophage cell line RAW264.7 [30]. This effect was attributed to the inhibition of the expression of iNOS mRNA induced by cytokines and endotoxins. Results from this study not only are in agreement with data obtained from a macrophage cell line, but also demonstrate that EGb did not impair cNOS activity in the same cells. Future studies of the selective effect of EGb in other cell types will be useful for a better understanding of the mechanisms of potential beneficial action of EGb. Compared with the standardized mixture of *Ginkgo biloba* extract EGb761, the extract used in this study contains the same amount of flavonoid glycosides (24%) and terpenoids (6%), which are regarded as the two major active constituents [25, 30]. It has been suggested that the combined activity and a

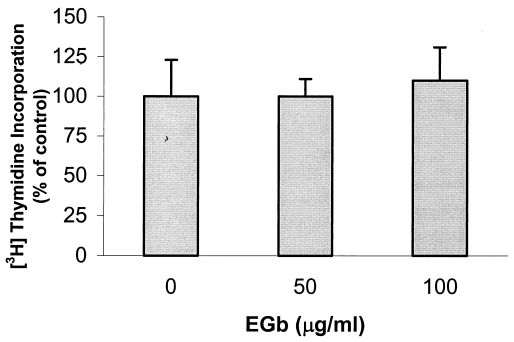

FIG. 9. Effect of EGb on the incorporation of [^{3}H]thymidine into endothelial cells. Cells were incubated with [^{3}H]thymidine in the presence or absence of EGb. After 4 hr of incubation, the radioactivity associated with the cells was determined. Results are expressed as a percentage of control, and each point represents the mean ± SD from three separate experiments. The control value was 14,670 ± 2,350 cpm/mg of cellular protein.

certain interdependency of several active constituents of the extracts are responsible for its beneficial effects [25]. In this study, the regulation of the iNOS mRNA level in endothelial cells may involve some or all of the active constituents of the extract.

In summary, results from this study have demonstrated clearly that EGb inhibited the production of NO in endothelial cells (ECV304). One of the mechanisms of this effect was via the attenuation of iNOS mRNA levels in these cells. This inhibitory effect on the synthase was calcium independent. On the other hand, EGb did not affect the activity of cNOS or the expression of cNOS mRNA in the same cells. Selective inhibition of iNOS by EGb may be therapeutically relevant for balancing NO production in endothelial cells in vascular disorders. It remains to be determined which components or metabolites of EGb are responsible for this effect.

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