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Ginkgo biloba Extract (EGb 761): Inhibitory Effect on Nitric Oxide Production in the Macrophage Cell Line RAW 264.7

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ABSTRACT. The present study was conducted to evaluate the effect of *Ginkgo biloba* extract (EGb 761) on the synthesis of nitric oxide (NO) induced by lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) in the mouse macrophage cell line RAW 264.7. EGb 761 inhibited nitrite and nitrate production, taken as an index for NO, in a concentration-dependent fashion. The IC_{50} for inhibition of nitrite production by activated macrophages was about 100 μg/mL EGb 761. The inducible NO synthase (iNOS) enzyme activity of cytosolic preparations from activated RAW 264.7 cells was inhibited by treatment with EGb 761. In addition, reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that the expression of iNOS mRNA in activated macrophages was suppressed by high concentrations of EGb 761. However, NF-κB DNA binding activity induced by activation with LPS/IFN-γ was not inhibited by EGb 761. These findings indicate that not only does EGb 761 directly act as an NO scavenger but also that it inhibits NO production in LPS/IFN-γ-activated macrophages by concomitant inhibition of induction of iNOS mRNA and the enzyme activity of iNOS. Thus, EGb 761 may act as a potent inhibitor of NO production under tissue-damaging inflammatory conditions. BIOCHEM PHARMACOL 53;6:897–903, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. *Ginkgo biloba* extract; EGb 761; nitric oxide; inducible nitric oxide synthase; macrophages; flavonoids

NO[‖] is synthesized from L-arginine by constitutive and inducible NOS in numerous mammalian cells and tissues [1]. To date, at least three NOS genes and proteins have been identified. Two enzymes, termed cNOS, are generally constitutive. These enzymes produce small amounts of NO over several minutes in response to stimuli that elevate intracellular Ca^{2+} [2]. The third isoform, termed iNOS, is generally inducible and is independent of changes in intracellular Ca^{2+} [2]. Macrophages [3], smooth muscle cells [4], endothelial cells [5], hepatocytes [6], and cardiac myocytes [7], as well as other cells having iNOS activity, produce high amounts of NO that are sustained for long periods when activated by stimuli including LPS, IFN-γ, interleukin-1, or tumor necrosis factor-α. The nanomolar concentrations of NO produced by cNOS are sufficient for intracellular signaling, while NO produced by iNOS in macrophages is a defense molecule with cytotoxic, cytostatic, microbicidal, and microbiostatic activities [8]. Moreover, in pathological conditions, macrophages can greatly increase their production of both NO and superoxide anion simultaneously, resulting in the formation of $ONOO^-$, which, through further reaction, can exert even stronger oxidant effects [9]. Therefore, high amounts of NO are potentially cytotoxic, capable of injuring the surrounding cells and tissues indiscriminately by itself or by formation of $ONOO^-$. Indeed, it has been reported that excess production of NO by macrophages and other cells exposed to endotoxin may contribute to septic shock [10]. $ONOO^-$ has been implicated as the injurious agent in cerebral injury [11] and myocardial ischemia [12]. Furthermore, it has been reported that $ONOO^-$ may contribute to atherosclerosis through oxidation of LDL within the arterial walls [13]. Thus, the inhibition of iNOS expression and/or activity represents an important therapeutic goal.

Extract leaves or fruits of *Ginkgo biloba* trees have been

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‖ *Abbreviations:* EGb 761, *Ginkgo biloba* extract; FBS, fetal bovine serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; LDL, low density lipoprotein; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; PCR, polymerase chain reaction; and RT-PCR, reverse transcription-polymerase chain reaction.

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used medicinally for hundreds of years. In particular, EGb 761, a defined extract prepared from the leaves of *Ginkgo biloba* according to a well-defined procedure (IPSEN Institute, Paris), has been effective in the treatment of disorders related to oxidative stress. In Europe, EGb 761 is commonly used to treat peripheral arterial diseases and cerebral insufficiency [14]. EGb 761 contains two groups as its main active constituents: 24% flavonoids (ginkgo-flavone glycosides; quercetin, kaempferol, isorhamnetin) and 6% terpenoids (ginkgolides, bilobalides) [14]. The combined activity of several constituents of *Ginkgo biloba* is likely responsible for the therapeutic benefit that is achieved. EGb 761 is a potent scavenger of free radicals, such as superoxide radical [15], hydroxyl [16], peroxyl [17], and NO [18] *in vitro*. Though we have demonstrated previously that EGb 761 can directly scavenge NO in *in vitro* systems [18], its effect on NO production in intact cells expressing iNOS and producing large amounts of NO is still unknown. To obtain further insight into the biological effects of EGb 761, in the present study we have investigated whether EGb 761 affects iNOS expression and activity in the mouse macrophage cell line RAW 264.7, stimulated with LPS plus IFN-γ.

MATERIALS AND METHODS

Reagents

Ginkgo biloba extract, EGb 761, was provided by IPSEN (Paris). IFN-γ was obtained from Genzyme (Cambridge, MA). FBS was obtained from the University of California, San Francisco cell culture facility. Oligonucleotide primers were obtained from Clontech (Palo Alto, CA). (6*R*)-5,6,7,8-Tetrahydrobiopterin was obtained from Alexis (San Diego, CA). L-[U-^{14}C]Arginine was obtained from DuPont (Wilmington, DE). Other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture

The murine monocyte/macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD). RAW 264.7 cells were cultured in 75 cm^2 plastic flasks (Falcon, NJ) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics. Cells were passed every 4 days. For experiments, macrophages were detached by vigorous pipetting and, after centrifugation, plated in fresh medium. These cells were activated with a combination of IFN-γ (10 U/mL) and LPS (10 ng/mL), and cultured for 20 hr at 37° in an atmosphere of 5% CO_2 plus air.

Deproteinization of Sample

The cultured medium was deproteinized following the method of Habu *et al.* [19]. After the macrophages were cultured, the supernatant from the plated cells (400 μL) was mixed with 290 μL of 0.3 M NaOH solution. After incubation for 5 min at room temperature, 290 μL of 5% (w/v) $ZnSO_4$ was added. The mixture was then allowed to stand for another 5 min, and was centrifuged at 2800 *g* for 20 min. The supernatant was filtered with a 0.45 μm nylon syringe filter.

Quantification of Nitrite and Nitrate

Nitrite and nitrate levels in the supernatant (100 μL) were quantified using the automated NOx analyser (model TCI-NOX 1000; Tokyo Kasei Kogyo, Tokyo, Japan), which employs the technique of automated flow injection analysis. Nitrite reacts with the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4), and forms a di-azo compound; the absorbance at 540 nm is measured with a flow-through visible spectrophotometer (model S/3250; Soma Kogaku, Tokyo, Japan) connected to a chart recorder. Nitrate was determined by reducing it to nitrite using an A7200 copperized cadmium reduction column (Tokyo Kasei Kogyo), and then quantified by nitrite as above. Both sodium nitrite and nitrate solution were used as standards.

RT-PCR

Total RNA was extracted from cultured cells following the method of Chomczynski and Sacchi [20]. RT was performed using an RNA PCR Kit (Perkin–Elmer, Branchburg, NJ). One microgram of total RNA was reverse transcribed to cDNA following the manufacturer's recommended procedures. RT-generated cDNA encoding iNOS and G3PDH (as a positive control and an internal standard) genes were amplified using PCR (35 cycles). Oligonucleotide primers which correspond to the mouse macrophage iNOS and murine G3PDH cDNA were purchased from Clontech and used following the manufacturer's procedures. PCR was also performed using an RNA PCR-Kit. The reaction volume was 40 μL containing (final concentration): PCR buffer (1×), deoxynucleotide (0.2 mM each), $MgCl_2$ (2 mM), Taq DNA polymerase (2 U), oligonucleotide primer (0.5 μM each), and RT products. After an initial denaturation for 2 min at 95°, 35 cycles of amplification (95° for 1 min, 65° for 1 min, and 72° for 1.5 min) were performed followed by a 7-min extension at 72°.

Analysis of PCR Products

A 10-μL aliquot from each PCR reaction was electrophoresed in a 1.7% agarose gel containing 0.2 μg/mL ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, PCR bands on the photograph of the gel were scanned using a laser densitometer linked to a computer analysis system. We normalized the iNOS signal relative to the corresponding G3PDH signal from the same sample, and data were expressed as the iNOS/G3PDH ratio.

Electrophoretic Mobility Shift Assay

Macrophages (1×10^6 cells/35 mm culture dish) were incubated with EGb 761 and/or LPS/IFN-γ as indicated in the figure legend. Nuclear extracts were isolated as described by Suzuki and Packer [21]. Binding reactions were performed for 20 min at 25° with 1 μg total protein in 20 μL of 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 2% glycerol, 1 μg poly(dI-dC), and ^{32}P-labeled κB probe labeled with T4 kinase and [γ-^{32}P]ATP. Proteins were separated by electrophoresis through a native 6% polyacrylamide gel in a running buffer of 12.5 mM Tris–borate, 0.25 mM EDTA (pH 8.0), followed by autoradiography.

MTT Assay for Cell Viability

RAW 264.7 cells were cultured in the presence of various concentrations of EGb 761 in a volume of 500 μL culture medium in 24-well tissue culture plates at 37° in 5% CO_2 plus air for 24 hr. The procedure used for the MTT assay was based on the method described by Mosmann [22]. MTT solution (0.5 mg/mL) was added to each culture well. After incubation for an additional 3 hr at 37°, the formazan crystals were dissolved by addition of 500 μL acid-isopropanol (0.04 N HCl in isopropanol). The absorbance of the solutions was read on a spectrophotometer, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

NOS Activity Assay

The enzyme preparation was obtained from RAW 264.7 cells (approximately 3×10^7 cells) activated with 50 U/mL IFN-γ and 5 μg/mL LPS for 16–18 hr. The cells were collected, washed in DMEM, and disrupted by five to six freeze–thaw cycles in 50 mM Tris–HCl, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 2 μM leupeptin, and 0.1% 2-mercaptoethanol. The lysate was centrifuged at 15,000 *g* for 30 min at 4°, and the supernatant was collected for NOS enzyme activity assay. NOS activity was assayed following [^{14}C]citrulline formation from [^{14}C]arginine, as described by Weinberg *et al.* [23] with minor modifications. Briefly, the reaction mixture (100 μL) contained 1 mM NADPH, 10 μM FAD, 1 mM dithiothreitol, 100 μM (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4), 10 μM L-arginine supplemented with L-[U-^{14}C]arginine (0.5 μCi/mL reaction medium) and 20 μL of supernatant in 50 mM HEPES (pH 7.5). After 60 min of incubation at 37°, the reaction was terminated by addition of 200 μL of cold-stop buffer (50 mM morpholino ethanesulfonic acid, pH 5.5, and 5 mM EDTA). The reaction mixture was then applied to a 0.5 mL column of Dowex 50W-X4 cation exchange resin (Na^+ form) from Fluka (Buchs, Switzerland), preequilibrated with stop buffer, which was eluted with 1 mL of stop buffer. Radiolabeled citrulline was quantified by using a liquid scintillation counter.

Statistical Analysis

All values are expressed as means ± SD. Student's unpaired *t*-test was used to assess the statistical significance of differences.

RESULTS

Effect of EGb 761 on Nitrite and Nitrite/Nitrate Production in Macrophages

The ability of EGb 761 to influence the NO production in RAW 264.7 macrophages was investigated. We analyzed accumulated nitrite and nitrate levels in the culture medium as an index for NO synthesis from these cells using Griess reagent, since NO is reactive in oxygenated aqueous solution and decomposes to nitrite and nitrate. It was confirmed that EGb 761, even at high concentrations, did not interfere with the reaction between nitrite and Griess reagent (data not shown). The efficacy of EGb 761 on nitrite or nitrite/nitrate production in macrophages is shown in Fig. 1. Nitrite and nitrite/nitrate production were dependent on the activation state of the cells. Unstimulated macrophages, after 20 hr of culture, produced the lowest levels of nitrite and nitrite/nitrate (1.93 ± 0.61 and 2.76 ± 0.83 μM, respectively). When these resting cells were incubated with EGb 761 alone, amounts of nitrite and nitrite/nitrate in the medium were maintained at a level similar to the unstimulated sample. A major increase of nitrite and nitrite/nitrate production was observed after treatment with LPS (10 ng/mL) plus IFN-γ (10 U/mL); these stable products increased 30-fold (56.7 ± 1.45 μM nitrite) and 50-fold (136.3 ± 7.2 μM nitrite/nitrate), respectively. In this experiment, macrophages were incubated with various con-

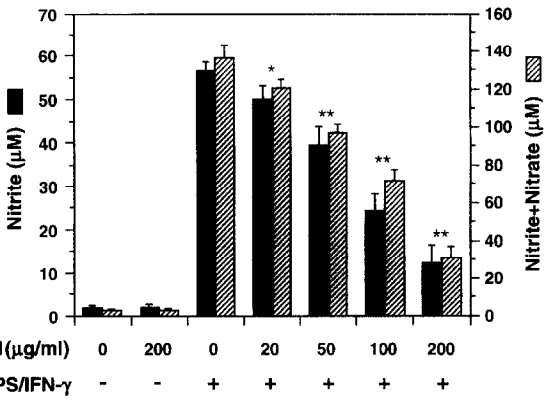

FIG. 1. Effect of EGb 761 on nitrite and nitrite/nitrate production in macrophages. RAW 264.7 macrophages (2.5×10^5 cells/0.5 mL/well) were incubated with medium alone or with LPS (10 ng/mL) plus IFN-γ (10 U/mL) in the presence of the indicated concentrations of EGb 761. After cells were incubated for 20 hr, to quantify the level of nitrite or nitrite/nitrate in the medium, cultured medium was analyzed both with and without the copperized cadmium column, as described in Materials and Methods. Each value is the mean ± SD of three independent experiments. Key: (*) $P < 0.05$ compared with the LPS/IFN-γ stimulation group; and (**) $P < 0.01$ compared with the LPS/IFN-γ stimulation group.

centrations of EGb 761 followed by activation with LPS/IFN-γ. The IC_{50} for inhibition of nitrite production was about 100 μg/mL. Moreover, EGb 761 suppressed nitrite/nitrate production in a manner similar to nitrite. When macrophages were cultured with 200 μg/mL EGb 761 for 20 hr, both nitrite and nitrite/nitrate production were inhibited by 80%. No effect on cell viability was detected, even in the presence of 200 μg/mL EGb 761, as measured by the MTT assay (data not shown). This result indicates that the inhibition of nitrite and nitrite/nitrate production by EGb 761 is not due to cell death.

Effect of EGb 761 on the Expression of iNOS mRNA in Macrophages

As shown in Fig. 2 (top panel), RAW 264.7 macrophages did not express detectable levels of iNOS mRNA after 7 hr of incubation with medium alone. In contrast, LPS/IFN-γ induced a dramatic increase in iNOS mRNA expression. EGb 761 (200 μg/mL) alone did not affect the basal expression of iNOS mRNA (data not shown). EGb 761 was added to the medium simultaneously with the stimuli. RT-PCR analysis of LPS/IFN-γ-activated macrophages treated with EGb 761 showed suppression of iNOS mRNA expression (Fig. 2). This inhibitory effect was significant at high concentrations of EGb 761, although slight inhibition of the expression was observed at lower concentrations. The maximal suppression on LPS/IFN-γ-induced iNOS mRNA expression was 41.0 ± 2.5% (200 μg/mL EGb 761) (Fig. 2, bottom panel). These results indicate that one factor that contributes to the inhibitory effect on NO production in macrophages is interference with the expression of iNOS mRNA by EGb 761.

Effect of EGb 761 on DNA Binding Activities of NF-κB

NF-κB is a transcription factor that is activated in response to stimulation by LPS, IFN-γ, and other agents. NF-κB activation is an essential factor in iNOS expression in macrophages; the involvement of NF-κB in iNOS expression is supported by its identification in the promoter region of the iNOS gene [24, 25]. To assess the effect of EGb 761 on early stages of iNOS gene expression, the activation of NF-κB in RAW 264.7 macrophages was examined using the electrophoretic mobility shift assay (Fig. 3). A nonspecific band was detected in nuclear extracts obtained from cells with or without treatment with LPS/IFN-γ. At 1 hr after activating with LPS/IFN-γ, the band of NF-κB was increased remarkably in nuclear extracts of macrophages. EGb 761 treatment along with LPS/IFN-γ did not affect the activation of NF-κB, even at the highest concentration (200 μg/mL), which had been shown to markedly inhibit the expression of iNOS mRNA. Consequently, it seems unlikely that EGb 761 directly modulates the activation of NF-κB, one of the significant stages for expression of iNOS enzyme.

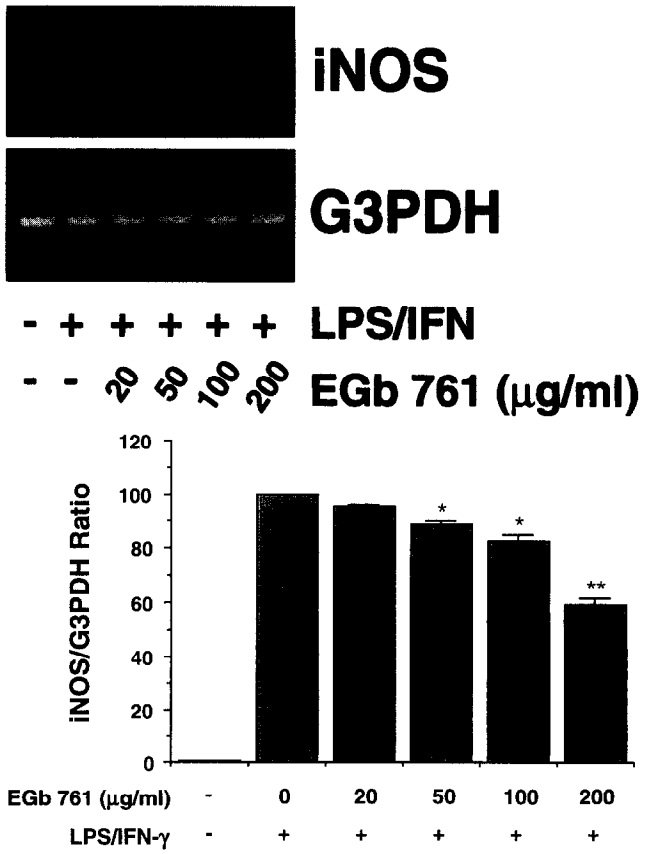

FIG. 2. Effect of EGb 761 on the expression of iNOS mRNA in macrophages. Top panel: Gel-photograph of PCR-amplified cDNA derived from iNOS and G3PDH mRNA. RAW 264.7 macrophages (2.5×10^6 cells/28 cm^2 culture dish) were incubated with medium alone or with LPS (10 ng/mL) plus IFN-γ (10 U/mL) in the presence of the indicated concentrations of EGb 761. After incubation for 7 hr, RNA was isolated using the acid guanidinium–phenol–chloroform method and then was subjected to RT-PCR analysis using specific primers for mouse macrophage iNOS and mouse G3PDH. Bottom panel: Densitometric analysis of the gel-photograph. The mRNA levels of iNOS and G3PDH on the gel-photograph were quantified by densitometry. All values are expressed as a percentage of the control for the iNOS/G3PDH ratio (ratio of iNOS to G3PDH) from results obtained by RT-PCR and represent the means ± SD of three independent experiments. Key: (*) $P < 0.05$ compared with the LPS/IFN-γ stimulation group; and (**) $P < 0.01$ compared with the LPS/IFN-γ stimulation group.

Effect of EGb 761 on Enzyme Activity of iNOS

To characterize the mechanism responsible for inhibition of nitrite production by EGb 761, we examined whether EGb 761 could directly affect the enzyme activity of NOS. NOS activity was determined by the conversion of radiolabeled arginine to citrulline using a cell-free cytosolic preparation from LPS/IFN-γ-activated RAW 264.7 cells. Figure 4 illustrates the inhibitory effect of EGb 761 on NOS activity. NOS activity was linear over at least 2 hr of incubation and over the range of protein concentrations used in these studies. Omission of the cofactors NADPH, FAD, and BH_4

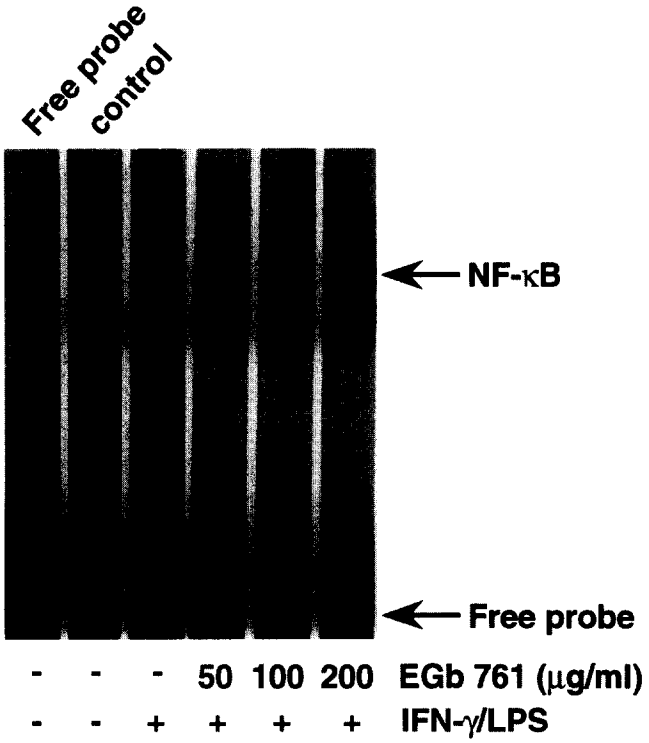

FIG. 3. Effect of EGb 761 on the DNA binding activity of NF-κB in activated macrophages. RAW 264.7 macrophages were incubated with medium alone or with LPS (10 ng/mL) plus IFN-γ (10 U/mL) in the presence of the indicated concentrations of EGb 761. After a 1-hr incubation, nuclear extracts were isolated, and an electrophoretic mobility shift assay was performed. Control indicates untreated cells (medium alone). One representative experiment is shown; similar results were obtained in three independent experiments.

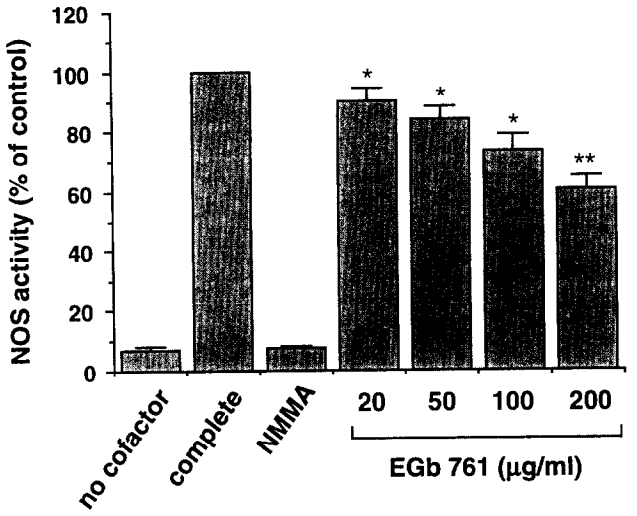

FIG. 4. Effect of EGb 761 on NOS activity. NOS activity was determined by the conversion of radiolabeled arginine to citrulline using a cytosolic preparation from macrophages. "No cofactors" indicates the absence of NADPH, BH_4, and FAD. "Complete" indicates that all constituents listed in Materials and Methods are contained in the reaction mixture. N^G-monomethylarginine (NMMA) was present in the assay at 100 μM. EGb 761 was added into the complete assay mixture as indicated. All values are expressed as a percentage of the control (100%: 205 ± 9 pmol/mg protein/min) and represent the means ± SD of three independent experiments. Key: (*) $P < 0.05$ compared with the LPS/IFN-γ stimulation group; and (**) $P < 0.01$ compared with the LPS/IFN-γ stimulation group.

resulted in 93% inhibition of NOS activity (Fig. 4). Addition of 100 μM N^G-monomethylarginine, a competitive inhibitor of NO synthesis, into the complete assay mixture inhibited the NOS activity by 92%. EGb 761 inhibited NOS activity in a concentration-dependent manner (39.4 ± 4.1% inhibition, 200 μg/mL EGb 761); however, the inhibitory effect was relatively small as compared with the extent to which nitrite production was inhibited. This result suggests that direct inhibition of NOS activity by EGb 761 is partially responsible for suppression of nitrite production.

DISCUSSION

EGb 761, an extract from *Ginkgo biloba* leaves, exhibits protective effects against biological free radicals in oxidative injury models, where it has been reported to scavenge the superoxide anion, hydroxyl radicals, peroxyl radical species, and NO directly [15–18]. NO produced *in vivo* has physiological roles, for example in the vasomotor effect of endothelial cells, but sustained production of high amounts of NO following the induction of iNOS has been implicated in the pathogenesis of important diseases such as atherosclerosis, circulatory shock, diabetes, chronic inflammation, and cancer, although the detailed molecular mechanisms are not clear. In atherosclerotic lesions, however, NO is thought to be important in the process by which LDL is oxidatively modified by reactive oxygen species (ROS) from macrophages. Moreover, increased generation of ROS by macrophages together with high amounts of NO production can lead to the formation of $ONOO^-$. Reactions of $ONOO^-$ have been proposed as a mechanism of tissue damage in a variety of pathologies.

Although we have demonstrated previously that EGb 761 can directly scavenge NO in *in vitro* systems [18], until now little was known about its effect on cellular systems. Besides the direct scavenging effect of NO, if EGb 761 can affect cytokine-mediated NO production from cells, this might substantially account for a therapeutic mechanism of EGb 761 against a variety of diseases. Hence, in this study, we examined the ability of EGb 761 to modulate NO production in the mouse macrophage cell line RAW 264.7.

It is well-established that NO spontaneously oxidizes to both nitrite and nitrate and that the ratio of these products is not constant. Activated macrophages can simultaneously produce superoxide and NO which react to form peroxynitrite, which decomposes exclusively to nitrate; therefore, in this experiment we quantified both nitrite and nitrate in the culture medium. EGb 761 inhibited nitrite and nitrate production from RAW 264.7 cells activated with LPS/IFN-γ in a similar concentration-dependent manner (Fig.

1). Its inhibitory effect was not due to cell death as assessed by the MTT assay.

NO production by macrophages is regulated by various factors (LPS, IFN-γ, transforming growth factor-β), and the induction of iNOS requires new protein synthesis [25]. Thus, NO produced by iNOS may be regulated at many sites, including transcription, post-transcription, translation, and post-translational modification [2]. The mechanism by which TGF-β negatively regulates iNOS in macrophages has been analyzed, and according to one report, its effect is caused by destabilizing iNOS mRNA, decreasing its translation, and increasing the degradation of iNOS protein [26]. For a complex product like EGb 761, which contains several active chemical constituents, many effects at various regulation sites are possible. We investigated the mechanism by which EGb 761 inhibited NO production from macrophages stimulated by LPS/IFN-γ.

We found that EGb 761 suppressed the level of iNOS mRNA in LPS/IFN-γ-activated macrophages, although the extent was not correlated with the inhibition of nitrite production. It is known that the expression of iNOS mRNA is regulated mainly at the transcriptional level [27]. The promoter region of mouse macrophages iNOS gene contains consensus sequences for binding of several transcription factors, including NF-κB, IFN-γ response element (γ-IRE), IFN regulatory factor-binding element (IRF-E), and nuclear factor-IL-6 (NF-IL-6) [24, 25, 28–30]. Furthermore, the presence of NF-κB binding sequences in the promoter of the iNOS and the requirement of translocation of NF-κB to the nucleus in the induction of iNOS have been demonstrated [25]. In our results, EGb 761 did not affect the DNA binding activity of NF-κB, although it inhibited the expression of iNOS mRNA. It is not clear that a 41% inhibition of the expression of iNOS mRNA would require comparable inhibition of NF-κB; however, it would be of interest to determine whether EGb 761 regulates LPS/IFN-γ-induced iNOS expression through other transcription factors. We also cannot exclude the possibilities that EGb 761 may decrease the stability of iNOS mRNA or affect translational regulation. Some flavonoids, including quercetin, a prominent ingredient of EGb 761, have been reported to inhibit protein kinase C [31], which is directly involved in the induction of NOS in rat hepatocytes [32]. In macrophages, it might be involved in the stabilization of iNOS mRNA expressed in response to IFN-γ stimulation [33]. This is another possible mechanism whereby EGb 761 suppresses iNOS mRNA in macrophages.

EGb 761 also inhibited NOS enzyme activity directly in intact cells, since suppression of nitrite production was observed even if EGb 761 was added 6 hr after cells were activated with LPS/IFN-γ, when iNOS mRNA had already been expressed (data not shown). Indeed, our results showed that EGb 761 directly inhibited iNOS enzyme from macrophage cytosolic fraction, as measured by the conversion of ^{14}C-labeled L-arginine to citrulline.

Taking these results together, EGb 761 seems likely to inhibit NO production by concomitant inhibitory actions, namely gene expression of iNOS, enzyme activity, and direct NO scavenging activity. EGb 761 inhibited NO production in LPS/IFN-γ-activated macrophages, with 57.3% (nitrite) and 47.7% (nitrite + nitrate) reduction at a concentration of 100 μg/mL, and an initial detectable effect at 20 μg/mL. These concentrations that inhibit NO production *in vitro* may be attained in plasma under therapeutic conditions [34]. When a dose of 360 mg/kg of radiolabeled EGb 761 was given to rats, 60% was absorbed. This is a whole-body concentration of about 220 μg/g. While this is admittedly a very large dose of EGb 761, it is also true that the extract will not be distributed evenly throughout the body; hence, some tissues and cellular compartments, especially plasma, will probably have a concentration in the range of 20–200 μg/mL, even at much lower doses of EGb 761. Thus, this may well be close to a physiological range. However, more pharmacokinetic studies must be done to verify this. In addition, it has been reported that the scavenging effect of EGb 761 on other free radicals was effective at these concentrations [17, 18]. EGb 761 consists of flavonoids, terpenoids, and some organic acids. Hence, the various constituents of EGb 761 have different pharmacological activities, and their additive or synergistic effects may be responsible for these inhibitory effects, although the exact mechanism is not understood. Further studies are needed to unravel the complex mechanism underlying the iNOS-inhibitory effect of EGb 761, especially its possible effects on gene transcription.

EGb 761 has beneficial effects in pathologies related to oxidative stress, such as ischemia and cardiac arrhythmia, probably through its free radical scavenging or transition metal binding properties. In the present study, we demonstrated that EGb 761 can also inhibit iNOS enzyme activity and gene expression. These findings expand the importance of EGb 761 as a multifunctional agent, and will help to clarify protection mechanisms of EGb 761 against various disorders. EGb 761 may also exert beneficial effects in inflammatory conditions that are accompanied by the expression of iNOS in various cells and tissues.

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