ELSEVIER

Biochemical Pharmacology 61 (2001) 503–510

Biochemical Pharmacology

Inhibition by ginkgolides and bilobalide of the production of nitric oxide in macrophages (THP-1) but not in endothelial cells (HUVEC)

Filly Cheung, Yaw L. Siow, Karmin O*

Department of Pharmacology, Institute of Cardiovascular Science and Medicine, Faculty of Medicine, The University of Hong Kong, 1/F, Li Shu Fan Building, 5 Sassoon Road, Pokfulam, Hong Kong

Received 4 January 2000; accepted 21 July 2000

Abstract

Nitric oxide (NO) is a principal mediator in many physiological and pathological processes. NO produced by constitutive nitric oxide synthase in endothelial cells (eNOS) acts as a vasodilator, whereas excess NO production due to elevated expression of inducible nitric oxide synthase (iNOS) may produce cytotoxic effects to cells in the vascular wall. We demonstrated in our previous work that the extract of *Ginkgo biloba* leaves (EGb) inhibits iNOS-mediated NO production. The objective of the present study was to investigate the effects of several active EGb components on iNOS-mediated NO production in macrophages derived from a human monocytic cell line (THP-1), as well as on eNOS-mediated NO production in human umbilical vein endothelial cells (HUVEC). Ginkgolide A, ginkgolide B, or bilobalide (0.25 to 1.0 μg/mL) caused a 30–65% reduction in the levels of NO metabolites released by THP-1 macrophages after 4 hr of incubation, with a corresponding decrease in iNOS activity. Western immunoblotting analysis coupled with a nuclease protection assay and reverse transcription–polymerase chain reaction revealed a concomitant reduction in the levels of iNOS protein mass and mRNA in ginkgolide A-, ginkgolide B-, or bilobalide-treated macrophages. On the other hand, these compounds did not affect eNOS-mediated NO production or the expression of eNOS protein and mRNA in HUVEC. Taken together, these results suggest that ginkgolide A, ginkgolide B, and bilobalide may contribute to the selective inhibitory effect of EGb on iNOS expression without affecting eNOS-mediated NO production.

Keywords: Nitric oxide; Nitric oxide synthase; Bilobalide; Ginkgolides; Macrophages; Endothelial cells

1. Introduction

The production of NO is regulated by NOS [1–3]. In the presence of co-substrates and co-factors, NOS converts L-arginine to NO and citrulline. The isoforms of NOS include endothelial constitutive NOS (eNOS or ecNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) [4,5]. Both eNOS and nNOS are constitutive types of NOS that require calcium for activation, whereas iNOS is calcium-independent [3–7]. NO derived from eNOS, in low levels, regulates physiological vasodilatation [8,9]. eNOS-catalyzed NO synthesis is impaired in atherosclerotic lesions and in cells treated with atherogenic factors, such as oxidized lipoproteins and homocysteine [9–13]. The iNOS is expressed mainly in macrophages, and its expression is regulated largely at the transcriptional level by many factors, such as various cytokines, growth factors, and endotoxins [14–18]. Upon induction, a high level of NO production can be sustained for a prolonged period of time. The excessive production of NO may lead to cell injury in the vascular wall [13,16,17]. Much attention has been paid to identifying factors that can selectively regulate individual types of NOS expression [18–20]. We reported previously that EGb selectively inhibits iNOS expression in cultured mammalian cells [21].

EGb has been used as a traditional herbal medicine for the treatment of cardiovascular and cerebral disorders [22–27]. Although this extract has been shown to affect NO production in various types of cells [21,24,25], the components or metabolites of EGb that are responsible for such an effect remain to be identified. Standardized EGb contains

* Corresponding author. Tel.: +852-2819-2861; fax: +852-2817-0859.

E-mail address: okarmin@hkucc.hku.hk (K. O).

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; eNOS, endothelial NOS; EGb, extracts of *Ginkgo biloba* leaves; THP-1, a human monocytic cell line; HUVEC, human umbilical vein endothelial cells; and RT–PCR, reverse transcription–polymerase chain reaction.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00567-0

24% flavonoid glycosides, 6% terpenoids, 7% proanthocyanidines, and other uncharacterized compounds [22]. Flavonoid glycosides and terpenoids are regarded as major active constituents of EGb [22]. Some components of EGb have been identified. For example, terpenoids consist of bilobalide and ginkgolides. Bilobalide accounts for about 2.9% of the total EGb (about 48% of terpenoids) [22]. Bilobalide was shown to have anti-ischemic properties and can prevent the hypoxia-induced decrease in ATP content in endothelial cells [28]. Both bilobalide and ginkgolide B have been suggested to possess an anti-apoptotic property [29]. Ginkgolide B was also shown to act as a specific platelet-activating factor (PAF) receptor antagonist [30,31]. It was suggested that the superoxide scavenging effect of ginkgolide B and bilobalide contributes to the antioxidant properties of EGb [32]. Although these studies support the notion that ginkgolides and bilobalide may have a protective effect on cardiovascular function, little information is available regarding the effect of these individual constituents of EGb on NO production. Because eNOS is expressed mainly in endothelial cells while iNOS is expressed mainly in macrophages, in the present study we investigated the effects of ginkgolide A, ginkgolide B, and bilobalide on iNOS-mediated NO production in THP-1-derived macrophages and on eNOS-mediated NO production in HUVEC. Ginkgolide A, ginkgolide B, and bilobalide were found to inhibit iNOS expression, leading to reduced NO production in THP-1 macrophages. However, these compounds did not affect the eNOS-mediated NO production in endothelial cells.

2. Materials and methods

2.1. Materials

Ginkgolide A, ginkgolide B, bilobalide (Sigma), and EGb (Shanghai Luyuan Industry Company Ltd.) [21] were dissolved in 5% ethanol (50 μg/mL). The final concentrations of ethanol present in the culture media (0.01 to 0.05%) had no effect on NO production in cultured cells. EGb is a standardized extract that contains 24% flavonoid glycosides and 6% terpenoids [21].

2.2. Cell culture

THP-1 cells, a human monocytic cell line, were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. For the experiments, cells were cultured in 35-mm dishes in the presence of phorbol 12-myristate 13-acetate (PMA) for 24 hr to induce differentiation of these cells into macrophage-like cells (THP-1 macrophages) [33]. THP-1 macrophages were activated with a combination of lipopolysaccharide (10 μg/mL) and tissue necrosis factor-α (0.1 μg/mL) for 12 hr. HUVEC were isolated from the umbilical vein by collagenase digestion [34,35], and were cultured in M199 medium supplemented with 10% fetal bovine serum and 30 mg/L of endothelial cell growth supplement (Sigma).

2.3. Determination of nitrite and nitrate levels in the culture medium

After cells were incubated with or without ginkgolide A, ginkgolide B, or bilobalide, the culture media were collected, and the amount of NO metabolites (nitrite and nitrate) was determined by a modified Griess reaction [21,36]. Briefly, the nitrate in the culture media was first converted to nitrite by the action of NADPH-dependent nitrate reductase. The Griess reaction was initiated by the addition of a reagent that contained 1% (w/v) sulfanilamide and 0.1% (w/v) *N*-[1-naphthyl]-ethylenediamine. The absorbency of the reaction mixture was read at 540 nm.

2.4. Determination of NOS activity

After THP-1 macrophages were treated with ginkgolide A, ginkgolide B, or bilobalide, the activity of iNOS was determined as described previously [21]. Briefly, the cells were lysed in 30 mmol/L of HEPES buffer (pH 7.4) containing 0.1 mmol/L of EDTA, 1 μmol/L of leupeptin, 2 mg/L of aprotonin, 1 mmol/L of dithiothreitol, 1 mmol/L of phenylmethylsulfonyl fluoride, and 0.1% (v/v) Triton X-100. The reaction mixture contained 30 mmol/L of HEPES buffer (pH 7.4), 1 mmol/L of EGTA, 100 μmol/L of NADPH, 300 μmol/L of tetrahydrobiopterin (BH_4), 1 mmol/L of dithiothreitol, 10 μmol/L of FAD, and 40 μmol/L of L-[^{3}H]arginine (37 kBq/reaction mixture) as substrate. The reaction was carried out at 37° for 1 hr, and was terminated by the addition of 200 μL of stop buffer containing 100 mmol/L of HEPES (pH 5.5). The mixture then was applied to a column of Dowex cation exchange resin to separate the reaction product, L-[^{3}H]citrulline, from L-[^{3}H]arginine. The eluent was collected, and the radioactivity associated with citrulline was determined by liquid scintillation counting.

2.5. Western immunoblotting analysis of NOS protein

The effects of ginkgolide A, ginkgolide B, and bilobalide on NOS protein mass were determined by western immunoblotting analysis [21]. Briefly, 100 μg of cellular protein from control or treated cells was separated by electrophoresis on a 7.5% SDS polyacrylamide gel. Proteins on the gel were then transferred to a nitrocellulose membrane. The specific NOS band was identified by either anti-eNOS or anti-iNOS antibodies (Transduction Laboratories), which recognized 140 kDa human eNOS and 130 kDa human iNOS, respectively. Horseradish peroxidase-conjugated secondary antibodies were used to develop the membranes. Bands corresponding to NOS proteins were identified by positive controls of purified NOS protein according to the

similarity in relative mobility on the gel. The specific NOS protein bands were visualized using ECL reagents (Amersham) and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst® version 1.1).

2.6. Nuclease protection assay and RT–PCR analysis

Total RNA was isolated from cultured THP-1 macrophages or HUVEC with TRIzol reagent according to the manufacturer's instructions (Life Technologies). For the nuclease protection assay, the isolated RNA (10 μg) was hybridized with ^{32}P-end-labeled iNOS or eNOS oligonucleotide probes overnight at 30° followed by nuclease digestion according to the manufacturer's instructions (Ambion Inc. USA) [37]. A positive control, 28S rRNA oligonucleotide probe was used as an internal control. After digestion, the protected fragments were resolved on a denaturing polyacrylamide gel (12%) containing 8 M urea and transferred to a filter paper that was later exposed to X-ray film. The bands corresponding to iNOS mRNA or 28S rRNA were analyzed using a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst® version 1.1). Values were expressed as relative expression of iNOS mRNA normalized to 28S rRNA levels. The levels of iNOS mRNA and eNOS mRNA were also determined by RT–PCR as described previously [21]. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to verify equal PCR product loading for each sample.

2.7. Statistical analysis

Results were analyzed using a two-tailed independent Student's *t*-test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of ginkgolides and bilobalide on NO production in THP-1 macrophages

THP-1 macrophages were incubated with ginkgolide A, ginkgolide B, or bilobalide at a concentration of 0.5 μg/mL for various time periods, and NO production was determined. As shown in Fig. 1, ginkgolide A, ginkgolide B, or bilobalide had an inhibitory effect on the amount of NO metabolites released from macrophages after 2, 4, or 8 hr of incubation with a maximum effect observed after 4 hr of incubation. Next, cells were incubated with various concentrations of ginkgolide A, ginkgolide B, or bilobalide for 4 hr. As shown in Fig. 2A, THP-1 macrophages treated with ginkgolide A at concentrations of 0.25, 0.5, or 1.0 μg/mL produced a significantly lower amount of NO metabolites than the control. Similar results were observed in THP-1 macrophages treated with ginkgolide B (Fig. 2B) or bilobalide (Fig. 2C).

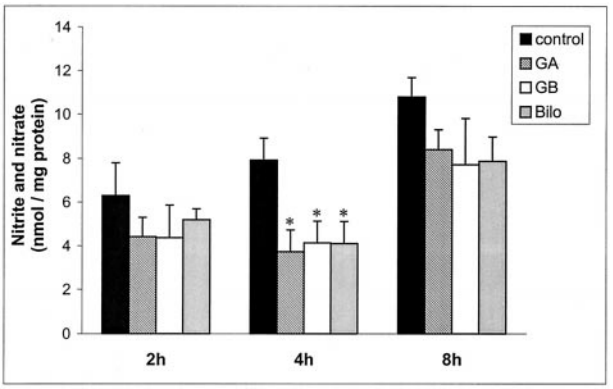

Fig. 1. Time course of the effects of ginkgolides and bilobalide on NO production in THP-1 macrophages. THP-1 macrophages were incubated in the absence (control) or the presence of ginkgolide A (GA, 0.5 μg/mL), ginkgolide B (GB, 0.5 μg/mL), and bilobalide (Bilo, 0.5 μg/mL) for various time periods. At the end of the incubation, culture media were collected, and aliquots were used for the determination of NO metabolite (nitrite and nitrate) levels. Results are expressed as means ± SD for five separate experiments. Key: (*) $P < 0.05$, when compared with the control values.

3.2. Effects of ginkgolides and bilobalide on NOS activities in THP-1 macrophages

Changes in the amount of NO metabolites released from cultured THP-1 macrophages may be due to a reduction in NOS activities in these cells. Since macrophages mainly express iNOS, NOS activity was determined without adding calcium into the reaction mixture. As shown in Fig. 3, ginkgolide A, ginkgolide B, or bilobalide at concentrations of 0.25, 0.5, and 1.0 μg/mL inhibited iNOS activity significantly.

3.3. Combined effects of ginkgolides and bilobalide on NO production in THP-1 macrophages

The combined effects of these compounds on NO production by THP-1 macrophages also were examined. As shown in Fig. 4, the combinations of (a) ginkgolide A and ginkgolide B, (b) ginkgolide A and bilobalide, (c) ginkgolide B and bilobalide, or (d) ginkgolide A, ginkgolide B, and bilobalide had an inhibitory effect on the release of NO metabolites from THP-1 macrophages, which was similar to the effects induced by the individual compounds. As a comparison, EGb at a concentration of 100 μg/mL displayed an inhibitory effect on NO production (Fig. 4).

3.4. Effects of ginkgolides and bilobalide on the expression of NOS protein in THP-1 macrophages

To examine whether a decrease in iNOS activity in THP-1 macrophages was a result of a change in the level of iNOS protein, a western immunoblotting analysis was performed. As shown in Fig. 5, treatment of THP-1 macrophages with ginkgolide A (0.5 μg/mL), ginkgolide B (0.5

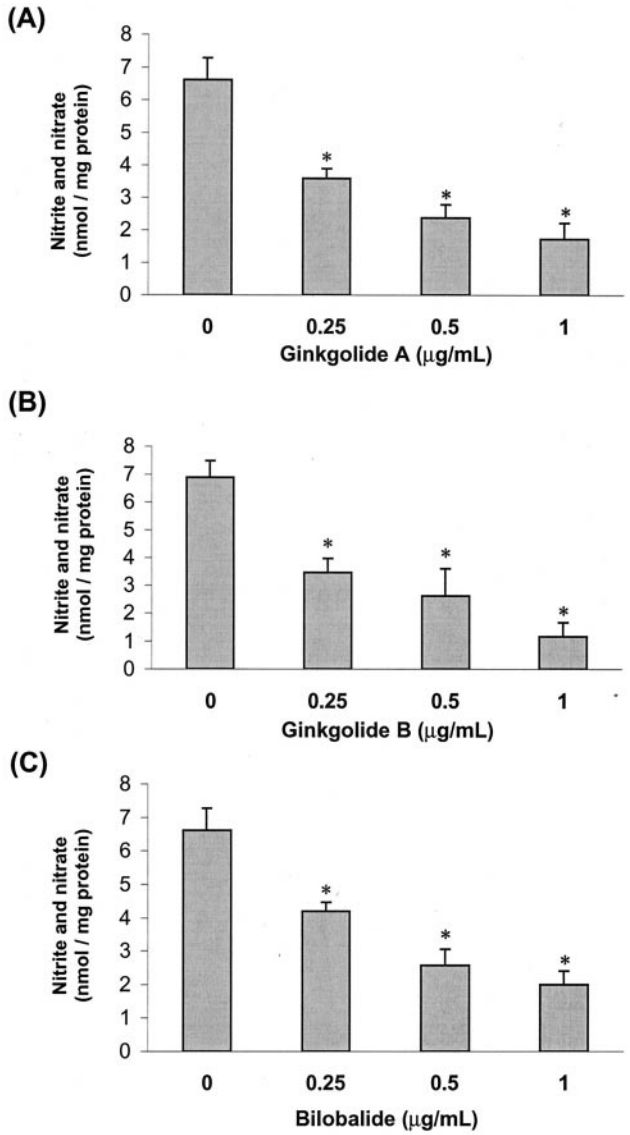

Fig. 2. Effects of ginkgolides and bilobalide at various concentrations on NO production in THP-1 macrophages. THP-1 macrophages were incubated in the absence (control) or the presence of (A) ginkgolide A, (B) ginkgolide B, or (C) bilobalide for 4 hr. At the end of the incubation, culture media were collected, and aliquots were used for the determination of NO (nitrate and nitrite) levels. Results are expressed as means ± SD for five separate experiments. Key: (*) $P < 0.05$, when compared with the control values.

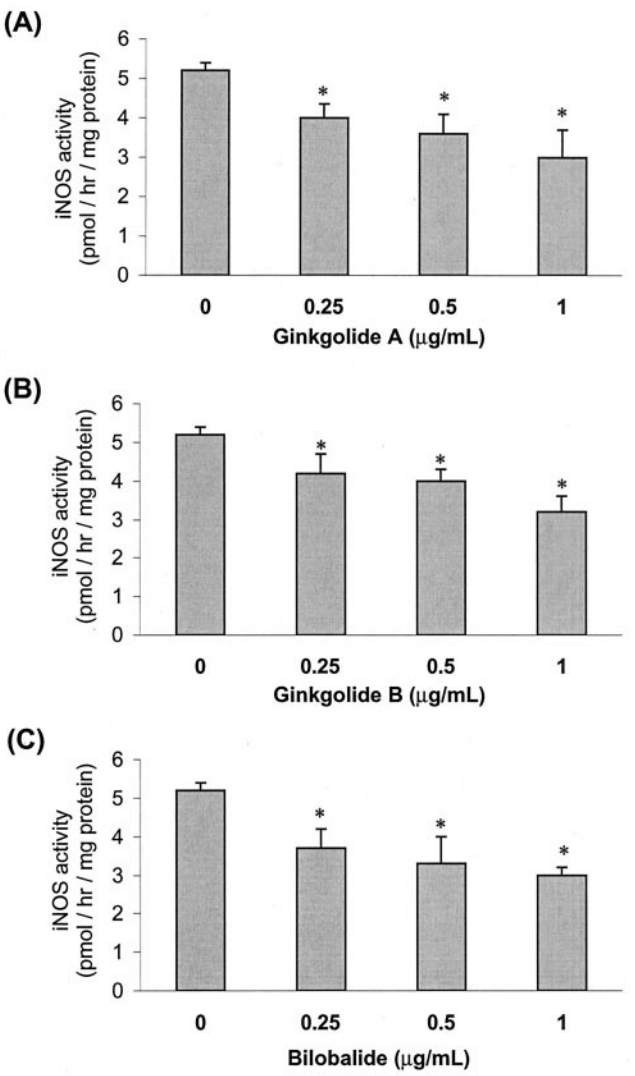

Fig. 3. Effects of ginkgolides and bilobalide on iNOS activity in THP-1 macrophages. THP-1 macrophages were incubated in the absence (control) or the presence of (A) ginkgolide A, (B) ginkgolide B, or (C) bilobalide for 4 hr. After incubation, the assay for iNOS activity was carried out in the absence of calcium. Results are expressed as means ± SD for five separate experiments. Key: (*) $P < 0.05$, when compared with the control values.

μg/mL), or bilobalide (0.5 μg/mL) caused a 54, 32, and 43% reduction in the levels of iNOS protein mass, respectively. EGb (100 μg/mL) also caused a significant reduction in iNOS protein mass. The eNOS protein was not detectable in THP-1 macrophages.

3.5. Effects of ginkgolides and bilobalide on the expression of NOS mRNA in THP-1 macrophages

To determine whether ginkgolide A, ginkgolide B, or bilobalide affected the expression of iNOS mRNA leading to a reduction in the iNOS protein level in THP-1 macrophages, a nuclease protection assay was performed. As shown in Fig. 6, ginkgolide A at a concentration of 0.5 μg/mL caused a significant reduction in the levels of iNOS mRNA. A similar inhibitory effect was observed when THP-1 macrophages were treated with ginkgolide B or bilobalide. For comparison, EGb at a concentration of 100 μg/mL also inhibited the expression of iNOS mRNA in these cells. The RT–PCR analysis also revealed an inhibitory effect of these compounds on the expression of iNOS mRNA (Fig. 6B). These results indicated that a reduction in the NO production in THP-1 macrophages treated with ginkgolide A, ginkgolide B, or bilobalide was due to reduced levels of iNOS mRNA.

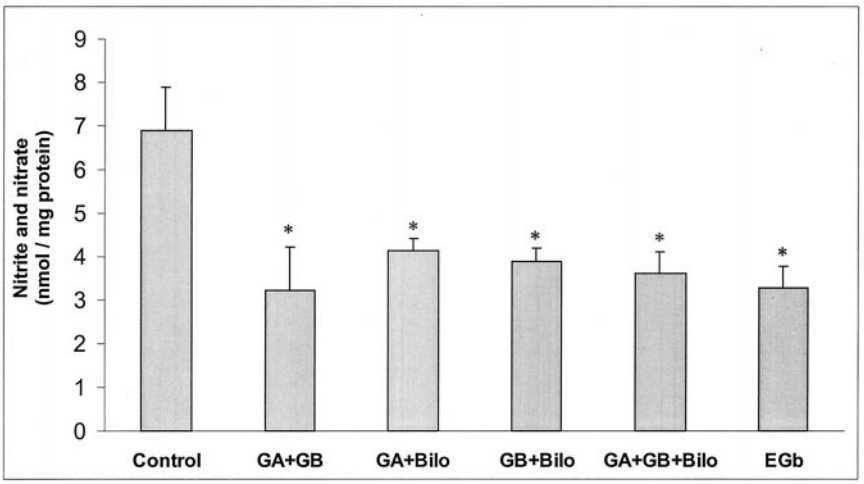

Fig. 4. Combined effects of ginkgolides and bilobalide or EGb on NO production in THP-1 macrophages. Cells were incubated in the absence (control) or the presence of ginkgolide A (GA, 0.5 μg/mL), ginkgolide B (GB, 0.5 μg/mL), and/or bilobalide (Bilo, 0.5 μg/mL) or EGb (100 μg/mL) for 4 hr. At the end of the incubation, culture media were collected, and aliquots were used for the determination of NO metabolite (nitrate and nitrite) levels. Results are expressed as means ± SD for three separate experiments. Key: (*) $P < 0.05$, when compared with the control values.

3.6. Effects of ginkgolides and bilobalide on NO production in HUVEC

To determine whether these compounds had any effect on eNOS-mediated NO production, similar experiments were performed in cultured HUVEC. As shown in Fig. 7A,

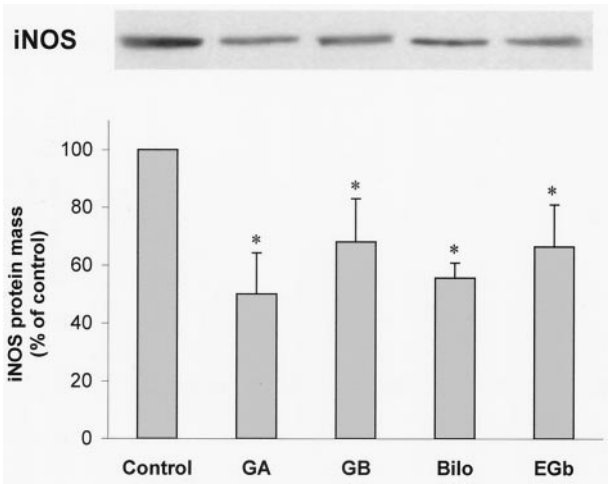

Fig. 5. Effects of ginkgolides and bilobalide on the expression of NOS proteins in THP-1 macrophages. THP-1 macrophages were incubated with ginkgolide A (GA, 0.5 μg/mL), ginkgolide B (GB, 0.5 μg/mL), bilobalide (Bilo, 0.5 μg/mL), or EGb (100 μg/mL) for 4 hr. In the control experiments, cells were cultured with medium alone. After the incubation, an equal amount of cellular proteins from control or treated cells was separated on a 7.5% SDS polyacrylamide gel followed by electrophoretic transfer of proteins from the gel to nitrocellulose membrane. (Top) The band of iNOS was identified by western immunoblotting analysis with anti-iNOS antibodies. (Bottom) The immunoblots were analyzed by densitometry, and the data were generated as integrated density units. Results are expressed as a percentage of the control and are depicted as means ± SD for three separate experiments. Key: (*) $P < 0.05$, when compared with the control values.

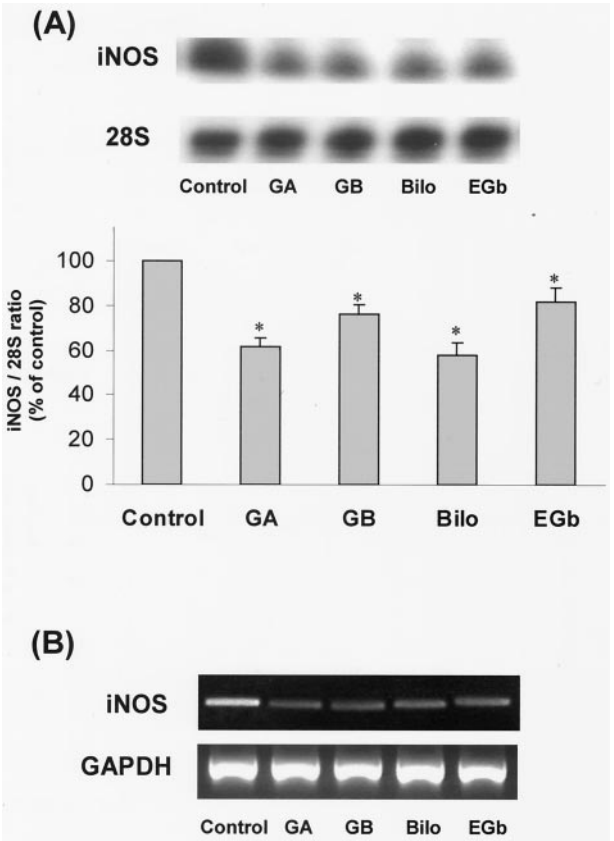

Fig. 6. Effects of ginkgolides and bilobalide on iNOS mRNA expression in THP-1 macrophages. After THP-1 macrophages were incubated with ginkgolide A (GA, 0.5 μg/mL), ginkgolide B (GB, 0.5 μg/mL), bilobalide (Bilo, 0.5 μg/mL), or EGb (100 μg/mL) for 4 hr, the expression of iNOS mRNA was determined by (A) the nuclease protection assay, and (B) RT–PCR. Cells without treatment were used as the control. Results are expressed as means ± SD from five separate experiments. Key: (*) $P < 0.05$, when compared with the control values.

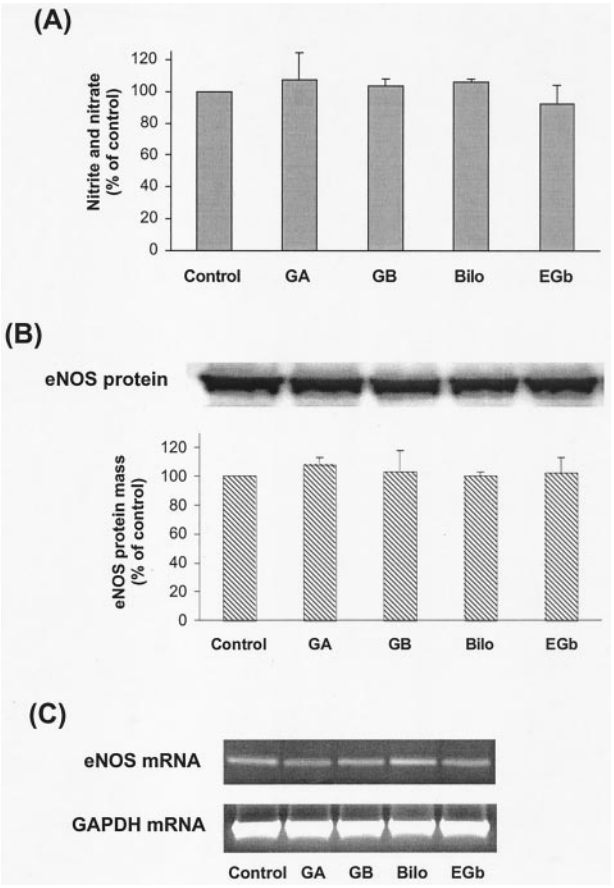

Fig. 7. Effects of ginkgolides and bilobalide on NO production and eNOS expression in HUVEC. Endothelial cells were incubated with ginkgolide A (GA, 0.5 μg/mL), ginkgolide B (GB, 0.5 μg/mL), bilobalide (Bilo, 0.5 μg/mL), or EGb (100 μg/mL) for 4 hr. At the end of the incubation, (A) the amounts of NO metabolites (nitrite and nitrate) released from these cells were measured. (B) The levels of cellular eNOS protein and (C) the levels of eNOS mRNA were determined by western immunoblotting analysis and RT–PCR, respectively. Results are expressed as means ± SD from five separate experiments.

ginkgolide A, ginkgolide B, or bilobalide treatment (0.5 μg/mL) did not alter the amount of NO metabolites released from HUVEC. Western immunoblotting analysis revealed that there was no change in the levels of eNOS protein in the treated HUVEC (Fig. 7B). Furthermore, these compounds had no effect on the expression of eNOS mRNA in HUVEC (Fig. 7C). The iNOS protein and mRNA were not detectable in HUVEC. As a comparison, the effect of EGb on NO production in HUVEC also was examined. As shown in Fig. 7, EGb (100 μg/mL) had no effect on NO production or on the expression of eNOS protein and mRNA in HUVEC.

4. Discussion

Although some beneficial effects of EGb against cardiovascular and cerebral disorders have been reported [22,23, 26], the contributions of its active constituents to these effects remain to be investigated. Results from our previous study suggest that the selective inhibitory effect of EGb on iNOS expression may be therapeutically relevant for balancing NO production in vascular disorders [21]. In the present study, we further investigated the effects of several individual constituents of EGb terpenoids on NO production in THP-1 macrophages and in endothelial cells. Our results clearly demonstrate that ginkgolide A, ginkgolide B, or bilobalide alone can inhibit NO production through the attenuation of iNOS mRNA expression in macrophages. These individual compounds (at a concentration of 0.5 μg/mL) exerted an inhibitory effect on NO production similar to that exerted by the EGb extract (at a concentration of 100 μg/mL) [21]. On the other hand, ginkgolide A, ginkgolide B, or bilobalide treatment did not affect eNOS-mediated NO production in endothelial cells.

It is well known that NO plays dual roles in physiological and pathological processes [1,2,8]. The eNOS-derived NO plays an important role in regulating vasorelaxation, while excessive production of NO by iNOS can lead to much cell damage in the vascular walls. The true therapeutic potential of NOS inhibition will only be defined with iNOS-selective inhibitors that do not affect eNOS activity [20]. In the present study, THP-1 macrophages were used as a model system to study the regulation of iNOS-mediated NO production, while HUVEC were used to study the regulation of eNOS-mediated NO production. The results obtained from the present study suggest that ginkgolide A, ginkgolide B, and bilobalide may have therapeutic implications for the treatment of disorders due to unbalanced (under or over) production of NO since all of those compounds can selectively inhibit iNOS-mediated NO production.

The flavonoid constituents in EGb, such as quercetin, were shown to have free radical scavenger as well as antioxidant properties [38,39]. A recent investigation also indicated that certain flavonoids inhibit NO production in lipopolysaccharide-activated macrophages, and such inhibitory activity may be due to a reduction of iNOS enzyme expression [40]. In the present study, we demonstrated that the terpenoids such as ginkgolide A, ginkgolide B, or bilobalide were able to inhibit NO production in cultured THP-1 macrophages by reducing iNOS mRNA expression. It was interesting to note that the combinations of ginkgolide A, ginkgolide B, and/or bilobalide did not exert a further inhibitory effect on NO production in THP-1 macrophages as compared with the effect of the individual compounds. These results suggest that ginkgolide A, ginkgolide B, and bilobalide might affect NO production in THP-1 macrophages via a similar pathway.

The present study demonstrated that individual terpenoids (ginkgolide A, ginkgolide B, and bilobalide) in the EGb extract are able to exert an inhibitory effect on the iNOS-mediated NO production similar to that of the EGb extract. As compared with EGb (100 μg/mL), much lower doses of the individual terpenoids (0.25 to 1.0 μg/mL) were required to induce this inhibitory effect. It is possible that

some components in EGb may exert opposite effects on NOS expression as compared with ginkgolide A, ginkgolide B, or bilobalide. Although ginkgolide A, ginkgolide B, and bilobalide are the major components in EGb terpenoids [22], the effects of other components in EGb on NO production mediated by eNOS and/or iNOS remain to be investigated.

In summary, this study clearly demonstrated that ginkgolide A, ginkgolide B, and bilobalide inhibit NO production in macrophages via attenuation of iNOS mRNA expression. These compounds have no effect on the eNOS-mediated NO production in endothelial cells. These components may play an important role in the EGb-mediated selective inhibition of iNOS expression in vascular walls.

Acknowledgments

The work described herein was supported, in part, by a grant from The University of Hong Kong Committee on Research and Conference Grants to Karmin O and by a grant from the Research Grant Council of Hong Kong (HKU 7297/98M and NSFC/HKU 39) and an Outstanding Researcher Award to Yaw L. Siow. We wish to thank Dr. T.T.H. Lao for the preparation of the HUVEC and Dr. G. Wang for technical help.

References

[1] Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109–42.
[2] Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem 1994;63:175–95.
[3] Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J 1994;298:249–58.
[4] Fleming I, Bauersachs J, Busse R. Calcium-dependent and calcium-independent activation of the endothelial NO synthase. J Vasc Res 1997;34:165–74.
[5] Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. J Biol Chem 1994;269:13725–8.
[6] Nathan C. Inducible nitric oxide synthase: what difference does it make? J Clin Invest 1997;100:2417–23.
[7] Cho HJ, Xie QW, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Nathan C. Calmodulin is a subunit of nitric oxide synthase from macrophages. J Exp Med 1992;176:599–604.
[8] Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. J Clin Invest 1998;101:731–6.
[9] Rubanyi GM. The role of endothelium in cardiovascular homeostasis and diseases. J Cardiovasc Pharmacol 1993;22:S1–14.
[10] Toborek M, Kaiser S. Endothelial cell functions. Relationship to atherogenesis. Basic Res Cardiol 1999;94:295–314.
[11] Upchurch GR, Welch GN, Loscalzo J. Homocysteine, EDRF, and endothelial function. J Nutr 1996;126:1290S–4S.
[12] Wever RM, Luscher TF, Cosentino F, Rabelink TJ. Atherosclerosis and the two faces of endothelial nitric oxide synthase. Circulation 1998;97:108–12.
[13] Buttery LD, Springall DR, Chester AH, Evans TJ, Standfield EN, Parums DV, Yacoub MH, Polak JM. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. Lab Invest 1996;75:77–85.
[14] Iwashina M, Hirata Y, Imai T, Sato K, Marumo F. Molecular cloning of endothelial, inducible nitric oxide synthase gene from rat aortic endothelial cell. Eur J Biochem 1996;237:668–73.
[15] Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992;256:225–8.
[16] Marczin N, Jilling T, Papapetropoulos A, Go C, Catravas JD. Cytoskeleton-dependent activation of the inducible nitric oxide synthase in cultured aortic smooth muscle cells. Br J Pharmacol 1996;118:1085–94.
[17] Gow AJ, Thom SR, Ischiropoulos H. Nitric oxide and peroxynitrite-mediated pulmonary cell death. Am J Physiol 1998;274:L112–8.
[18] Fukuto JM, Chaudhuri G. Inhibition of constitutive and inducible nitric oxide synthase: potential selective inhibition. Annu Rev Pharmacol Toxicol 1995;35:165–94.
[19] Furfine ES, Harmon MF, Paith JE, Knowles RG, Salter M, Kiff RJ, Duffy C, Hazelwood R, Oplinger JA, Garvey EP. Potent and selective inhibition of human nitric oxide synthases. Selective inhibition of neuronal nitric oxide synthase by *S*-methyl-L-thiocitrulline and *S*-ethyl-L-thiocitrulline. J Biol Chem 1994;269:26677–83.
[20] Griffiths MJ, Messent M, MacAllister RJ, Evans TW. Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br J Pharmacol 1993;110:963–8.
[21] Cheung F, Siow YL, Chen WZ, O K. Inhibitory effect of *Ginkgo biloba* extract on the expression of inducible nitric oxide synthase in endothelial cells. Biochem Pharmacol 1999;58:1665–73.
[22] DeFeudis FV. *Ginkgo biloba* extract (EGb 761): pharmacological activities and clinical applications. Paris: Elsevier, 1991.
[23] Mouren X, Caillard Ph, Schwartz F. Study of the antiischemic action of EGb761 in the treatment of peripheral arterial occlusive disease by $TcPo_2$ determination. Angiology 1994;45:413–7.
[24] Kobuchi H, Droy-Lefaix MT, Christen Y, Packer L. *Ginkgo biloba* extract (EGb 761): inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7. Biochem Pharmacol 1997;53:897–903.
[25] Varga E, Bodi A, Ferdinandy P, Droy-Lefaix MT, Blasig IE, Tosaki A. The protective effect of EGb 761 in isolated ischemic/reperfused rat hearts: a link between cardiac function and nitric oxide production. J Cardiovasc Pharmacol 1999;34:711–7.
[26] Pietri S, Maurelli E, Drieu K, Culcasi M. Cardioprotective and antioxidant effects of the terpenoid constituents of *Ginkgo biloba* extract (EGb 761). J Mol Cell Cardiol 1997;29:733–42.
[27] Janssens D, Remacle J, Drieu K, Michiels C. Protection of mitochondrial respiration activity by bilobalide. Biochem Pharmacol 1999;58:109–19.
[28] Janssens D, Michiels C, Delaive E, Eliaers F, Drieu K, Remacle J. Protection of hypoxia-induced ATP decrease in endothelial cells by *Ginkgo biloba* extract and bilobalide. Biochem Pharmacol 1995;50:991–9.
[29] Ahlemeyer B, Mowes A, Krieglstein J. Inhibition of serum deprivation- and staurosporine-induced neuronal apoptosis by *Ginkgo biloba* extract and some of its constituents. Eur J Pharmacol 1999;367:423–30.
[30] Bourgain RH, Andries R, Esanu A, Braquet P. PAF-acether induced arterial thrombosis and the effect of specific antagonists. Adv Exp Med Biol 1992;316:427–40.
[31] Cheng D, Chen W. Effects of ginkgolide B on isobaric hypoxic pulmonary hypertension in rats. Chin Med J (Engl) 1996;109:881–4.
[32] Scholtyssek H, Damerau W, Wessel R, Schimke I. Antioxidative activity of ginkgolides against superoxide in an aprotic environment. Chem Biol Interact 1997;106:183–90.
[33] Kurosaka K, Watanabe N, Kobayashi Y. Production of proinflammatory cytokines by phorbol myristate acetate-treated THP-1 cells and

monocyte-derived macrophages after phagocytosis of apoptotic CTLL-2 cells. J Immunol 1998;161:6245–9.

[34] Wong JT, Tran K, Pierce GN, Chan AC, O K, Choy PC. Lysophosphatidylcholine stimulates the release of arachidonic acid in human endothelial cells. J Biol Chem 1998;273:6830–6.

[35] Chow K, Cheung F, Lao TTH, O K. Effect of homocysteine on the production of nitric oxide in endothelial cells. Clin Exp Pharmacol Physiol 1999;26:817–8.

[36] Schmidt HHHW, Kelm M. Determination of nitrite and nitrate by the Griess reaction. In: Feelisch M, Stamler JS, editors. Methods in nitric oxide research. New York: John Wiley, 1996. p. 491–8.

[37] Nakagawa T, Nozaki S, Nishida M, Yakub JM, Tomiyama Y, Nakata A, Matsumoto K, Funahashi T, Kameda-Takemura K, Kurata Y, Yamashita S, Matsuzawa Y. Oxidized LDL increases and interferon-γ decreases expression of CD36 in human monocyte-derived macrophages. Arterioscler Thromb Vasc Biol 1998;18:1350–7.

[38] Ramassamy C, Naudin B, Christen Y, Clostre F, Costentin J. Prevention by *Ginkgo biloba* extract (EGb 761) and trolox C of the decrease in synaptosomal dopamine or serotonin uptake following incubation. Biochem Pharmacol 1992;44:2395–401.

[39] Oyama Y, Fuchs PA, Katayama N, Noda K. Myricetin and quercetin, the flavonoid constituents of *Ginkgo biloba* extract, greatly reduce oxidative metabolism in both resting and Ca^{2+}-loaded brain neurons. Brain Res 1994;635:125–9.

[40] Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochem Pharmacol 1999;58:759–65.