ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Inhibitory effects of *Ginkgo biloba* extract on vascular endothelial growth factor in rat aortic endothelial cells[1]

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KEY WORDS *Ginkgo biloba*; vascular endothelium; endothelial growth factors; lysophosphatidylcholines; enzyme-linked immunosorbent assay; *in situ* hybridization

ABSTRACT

AIM: To study the protective effects of *Ginko biloba* extract (*Gb*E) against rat aortic endothelial cells (RAEC) damage induced by lysophosphatidylcholine (LPC). **METHODS:** Cell injury were determined by MTT assay and LDH release. Vascular endothelial growth factor (VEGF) protein production from RAEC was determined by enzyme-linked immunosorbent assay (ELISA). VEGF mRNA expression was examined by *in situ* hybridization and dot blot. **RESULTS:** *Gb*E 0.01-1 μg/L prevented LPC-induced injury in cultured RAEC in a concentration-dependent manner. Cultured RAEC could express VEGF protein and VEGF mRNA was induced by LPC 5 mg/L. *Gb*E could inhibit the expression of VEGF protein and VEGF mRNA in co-cultured RAEC with LPC. **CONCLUSION:** LPC could induce a strong expression of VEGF in RAEC. *Gb*E could protect RAEC against the LPC-induced damage and downregulate VEGF protein and VEGF mRNA expression in cultured RAEC.

INTRODUCTION

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) is a heparin-binding, homodimeric glycoprotein (M_r=46 000) belonging to the platelet-derived growth factor (PDGF) family[1]. VEGF/VPF is an important regulator of vasculogenensis, angiogenesis, and vascular permeability *in vivo*[2]. It exerts its biological function through two high-affinity tyrosine kinase receptors expressed predominantly by endothelial cells (EC): vascular endothelial growth factor receptors (VEGFR), on the cellular membrane, namely, the kinase insert domain-containing receptor (KDR) and the fms-like tyrosine kinase-1 receptor (Flt-1). The murine homologue of KDR is also known as fetal liver kinase-1 receptor (Flk-1)[3].

Recent findings indicate that oxidized low density lipoprotein (Ox-LDL) may play a key role in the atherosclerotic process[4,5]. Lysophosphatidylcholine (LPC), which is increased in the plasma of hypercholesterolemic patients, is a component of Ox-LDL, and as such, may play an important role in atherosclerosis[6]. Endothelial dysfunction has been implicated in the pathogenesis of many cardiovascular disease, and has been shown to be a characteristic feature of atherosclerosis. VEGF is specific for EC and could stimulate EC proliferation *in vitro*[7]. Although VEGF may be involved in the development of atherosclerosis in humans.

*Gb*E is a potent scavenger of several reactive oxygen species such as single oxygen, superoxide anions,

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Received 2001-10-08 Accepted 2002-06-14

and hydroxyl radicals[8,9]. *Gb*E protected the heart against ischemia reperfusion damage and endothelial dysfunction evoked by oxygen free radicals[10,11]. The aim of study was to understand the effects of LPC on VEGF protein and VEGF mRNA expression in cultured RAEC and the protective effects of *Gb*E.

MATERIALS AND METHODS

RAEC culture RAEC were isolated from male Wistar rat (100-150 g) by the primary explants technique as described previously[12]. RAEC were incubated in 50 mL cultured flask at 37 ℃ in a 5 % CO_2 and 95 % ambient air environment. Culture medium was 50 % Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 20 % fetal bovine serum (FBS; Gibco), penicillin 100 kU/L, streptomycin 100 kU/L, and amphotericin B 0.25 mg/L, and the culture media were changed every 3 d. During the cells were grown into a 90 % confluent state, the cells were subcultured using 0.15 % trypsin (Sigma). In the experiments, RAEC at confluence were pretreated with serum-free DMEM for 12 h. The medium was then replaced with fresh, serum-free medium containing LPC 5 mg/L (Sigma) or LPC+*Gb*E (0.01, 0.1, and 1 μg/L) and incubated with cells for 24 h. *Gb*E was presented by Prof CHEN Wei-Zhou (Shanghai Institute of Material Medica). The total content of ginkgo flavonetrin more than 24 % and ginkgo lactone more than 6 %.

VEGF protein quantification RAEC were stimulated with LPC 5 mg/L or LPC 5 mg/L+*Gb*E (0.01, 0.1, and 1 μg/L) for 24 h at 37 ℃ in a 5 % CO_2 and 95 % ambient air environment. After incubation of RAEC as indicated, the conditioned medium was collected and centrifuged at 1000×*g* for 5 min, and the supernatant was used for the measurement of VEGF protein by enzyme-linked immunosorbent assay (ELISA) as described previously[16].

ELISA Culture supernatant 100 μL was dispensed into the plate, then 25 μL of reconstituted rat anti VEGF antibody (Santa Cruz) 4 mg/L was added to the plate and incubated overnight at 4 ℃. Then goat anti-rat IgG-HRP (SABC) was added into each well and incubated at room temperature for 30 min. After washing the plate 5 times with phosphate-buffered saline (PBS, pH 7.2), 100 μL *O*-phenylenediamine (OPD) containing 0.01 % H_2O_2 was added at room temperature and incubated for 25 min. Absorbance (*A*) was measured at 492 nm.

MTT assay RAEC suspensions 100 μL containing 1000 cells was added into each well of a 96-well Corning microtiter plate[12]. The plate were incubated for 3 h at 37 ℃ in a humidified 95 % air/5 % CO_2 atmosphere. Then cells were incubated with LPC 5 mg/L or LPC 5 mg/L+*Gb*E (0.01, 0.1, and 1 μg/L) for 24 h. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 5 g/L 20 μL was added to each well and the plates were incubated at 37 ℃ for 4 h. Cells were then lysed by incubation at room temperature for 20 min with 100 μL isopropanol (containing HCl 0.1 mol/L). Absorbance was read at room temperature at 570 nm.

LDH release LDH release were measured in the same cultures. LDH activity in the 20 μL extracellular medium was quantified by LDH reagent for clinical diagnosis (Nanjing Jiancheng Bioengineering Institute).

***In situ* hybridization analysis** Slips were placed into 35-mm culture dishes (Nalge Nunc International) with the RAEC. After incubating with serum-free DMEM for 12 h, the cells were stimulated by LPC 5 mg/L or LPC (5 mg/L)+*Gb*E (1 μg/L) for 12 h, in the control dish only DMEM was added. The sections were immersed in 4 % paraformaldehyde for 5 min and washed with PBS. The sections were then subjected to *in situ* hybridization as detailed in Brown *et al*[13]. The VEGF cRNA probe was purchased from Santa Cruz. The probe was marked and detected with Digoxin detection kit (Boehringer Mannheim).

RNA extraction and dot blot analysis When RAEC reached confluence, they were incubated with serum-free DMEM for 12 h. The cells were stimulated with LPC 5 mg/L or LPC 5 mg/L+*Gb*E 1 μg/L for 24 h. Total cultured RAEC RNA was isolated by Tissue/Cell Total RNA Isolation Kit (Watson, Shanghai) and 20 μg total RNA sample was analyzed by dot blot assay. Digoxin-labeled VEGF cDNA (obtained from Prof CHEN Xin-Sheng, University of Pennsylvania) was used as the probe. Total RNA samples were heated at 65 ℃ in 50 % formamide deioinizate, formaldehyde 2.2 mmol/L, and 1×MOPS. After placed in ice for 5 min, samples were blotted on a nitrocellulose membrane. Membrane was prehybridized for 3 h at 42 ℃ in 50 % formamide deioinizate, 5×SSPE, 5×Denhardt, 1 % sodium dodecyl sulfate (SDS), and ssDNA 200 mg/L, then hybridizationed with 30 ng digoxin-labeled VEGF cDNA probe for 20 h, as previously described. The membrane was washed and exposed to 80 ℃ for 2 h, then detected with Digoxin Detection Kit (Boehringer

Mannheim). The dot blot films were analyzed with smark view software (Furi Co). The corrected density with the software was plotted as a percentage of control value.

Statistical methods Each experiment was repeated at least three times. Data were presented as mean±SD. Differences were considered statistically significant when $P<0.05$ as analyzed by paired *t*-test.

RESULTS

Effect of LPC on VEGF protein levels in a conditioned medium After cells were incubated with LPC 5 mg/L for 24 h, VEGF production from RAEC was increased. The absorbance of LPC group was 0.235±0.006 ($P<0.01$ *vs* control group, $n=3$). *Gb*E (0.01, 0.1, and 1 μg/L) down-regulated VEGF production from RAEC in a concentration-dependent manner (Fig 1).

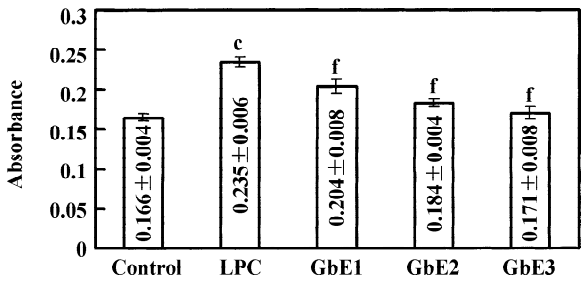

Fig 1. Inhibitory effect of *Gb*E on VEGF protein levels induced by LPC. *Gb*E1 was 0.01 mg/L, *Gb*E2 was 0.1 mg/L, and *Gb*E3 was 1 mg/L. $n=3$. Mean±SD. $^cP<0.01$ *vs* control group. $^fP<0.01$ *vs* LPC group.

MTT Assay LPC inhibited the growth of RAEC, and the inhibitory rate was 70.3 %. After coincubated with *Gb*E (0.01, 0.1, and 1 μg/L), the cell proliferation was increased (Tab 1).

LDH release The exposure of RAEC to LPC for 24 h produced a reduction in the cell viability. When the cells were cocultured with *GbE*, the LPC-induced cell damage was greatly attenuated in a concentration-dependent manner (Fig 2).

***In situ* hybridization** After exposure of RAEC to LPC 2.5 mg/L, the staining was stronger than control group. But it was weaker in LPC+*Gb*E 1 μg/L group than that in LPC group (Fig 3).

Dot blot hybridization There was no staining of VEGF mRNA in cultured RAEC, but there was strong staining after exposure to LPC 5 mg/L. After incubated with LPC 5 mg/L +*Gb*E 1 μg/L for 24 h, VEGF mRNA expression was lowered. The score of density and peak was reduced by 63.63 % over the LPC group.

Tab 1. Effect of *Gb*E on inhibition of growth by LPC in RAEC. $n=6$. Mean±SD. $^cP<0.01$ *vs* control group. $^fP<0.01$ *vs* LPC group.

Group	Concentration/ μg·L^{-1}	*A*	Inhibitory rate/%
Control group		0.072±0.004	
LPC group	5000	0.0213±0.0012^c	70.3
*Gb*E group	1	0.0610±0.0027^f	15.0
	0.1	0.034±0.004^f	53.3
	0.01	0.0325±0.0027^f	54.7

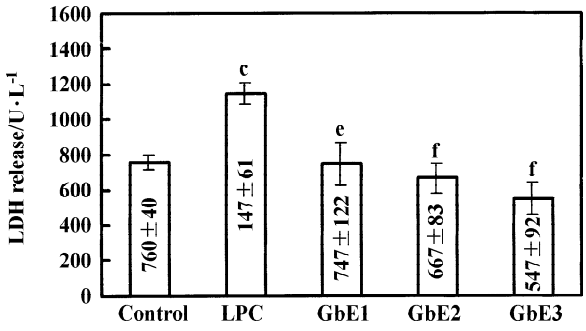

Fig 2. Effect of *Gb*E on LPC-induced LDH release in RAEC. *Gb*E1 was 0.01 mg/L, *Gb*E2 was 0.1 mg/L, and *Gb*E3 was 1 mg/L. $n=3$. Mean±SD. $^cP<0.01$ *vs* control group. $^eP<0.05$, $^fP<0.01$ *vs* LPC group.

DISCUSSION

The major hazardous elements of atherosclerosis involved abnormal blood-lipid metabolization, hypertension, diabetes, and so on, but the mechanism of atherosclerosis was not entirely proved. During the process of atherosclerosis, VEGF were formed and released from endothelial cells, smooth muscle cells, and macrophages within the lesions[14]. VEGF seem to play an important role in the progression of atherosclerosis.

LPC, a component of Ox-LDL, was demonstrated to upregulate VEGF mRNA expression in human umbilical veins endothelial cell line (ECV304) and to stimulate VEGF protein secretion from the cells in a time- and concentration-dependent manner in our previously

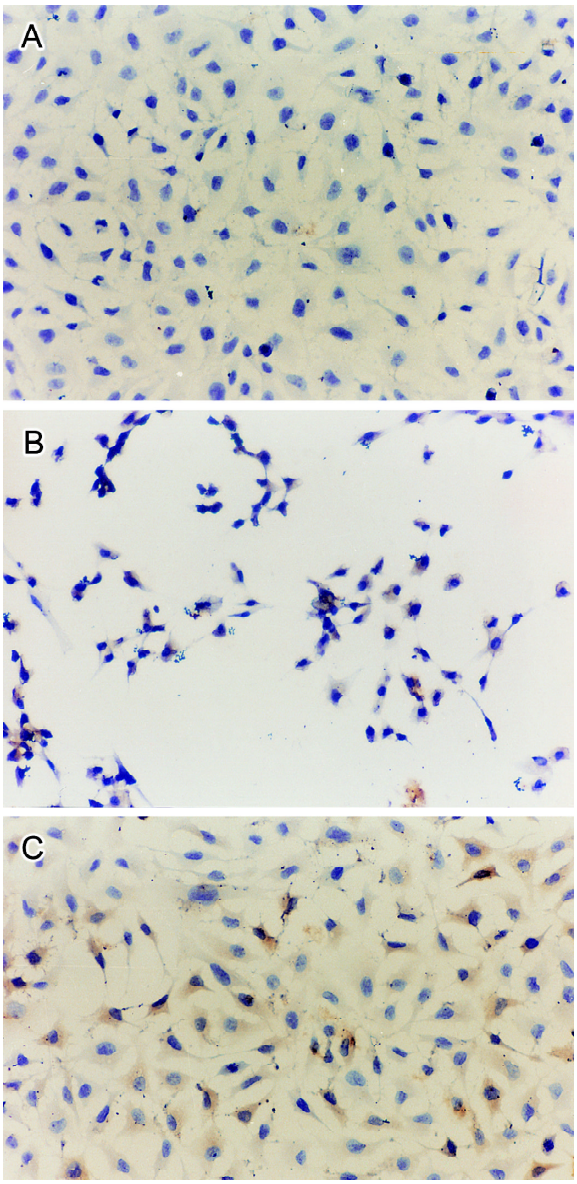

Fig 3. Photomicrograph of VEGF mRNA after stimulated with LPC and *Gb*E by *in situ* hybridization analysis. A: RAEC control; B: LPC 2.5 mg/L; C: LPC 2.5 mg/L+*Gb*E 1 mg/L. ×400.

studies[15,16]. In cultured RAEC, there was no *per se* staining for VEGF mRNA. A strong staining for VEGF mRNA was observed after stimulation by LPC, a weak staining for VEGF mRNA was observed after incubated with LPC and *Gb*E. Chen *et al*[17] demonstrated that *Gb*E protected rabbit endothelial cells against LPC-induced damage. In our studies, *Gb*E prevented the LPC-induced proliferation in cultured RAEC in a concentration-dependent manner.

In summary, we demonstrated that LPC induced VEGF protein and VEGF mRNA expression in cultured RAEC, and *Gb*E possessed a protective effect on RAEC against injury elicited by LPC and inhibited the expression of VEGF protein and VEGF mRNA. Our findings suggest that interaction between LPC and VEGF may be involved in the development of human atherosclerosis and *Gb*E may contribute to the prevention and treatment of atherosclerosis.

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