中國醫藥大學物理治療學系復健科學碩士班

碩 士 論 文

銀杏萃取物調降 LOX-1 所媒介之內皮細胞傷：活性氧自由基，MAPK 激酶及 NF-κB 之角色

Ginkgo biloba extract down-regulate LOX-1-mediated endothelial dysfunction: Role of ROS ,MAPK and NF-κB

指導教授：李信達 博士（Lee,Shin-Da PH.D）

共同指導教授: 歐秀中 博士（Ou,Hsiu-Chung PH.D）

研究生：陳凱玲 撰（Chen,Kai-Ling）

中 華 民 國 99 年 7 月

誌 謝

對我而言，兩年在人生中其實不長，但這七百多個日子教會了我好多，也讓我的人生有所成長！這些日子我開心過、笑過；難過過、哭過；想放棄過，但也撐過！謝謝毆老師的嚴格要求，讓歐家出品是品質保證！謝謝李老師指導我的論文，謝謝永華姐幫我們處理瑣碎雜事，謝謝伯昌老師、悅齡的關心以及鼓勵！太多的感謝難以用言語形容!

謝謝昆霖學長不厭其煩的教我實驗，解答我的問題，謝謝你對這小又很溫暖的實驗室付出，謝謝你為我擔了這麼多，祝你在未來的學術領域，因著做實驗而快樂！謝謝凡妮、憶帆我們有難忘的美國之旅，不管妳們今後會在哪裡，我會永遠想念妳們！謝謝泳璁、智超、曉玲陪我度過難忘的聖誕節，這樣的景象已不再會有，但那是我們一起有溫馨冬天的回憶，祝你們前程似錦！謝謝云禎、珮慈、群超、畢憶、雅晴對於實驗所需的幫忙！我不會忘記，晚上十一點被關在電梯中庭的我，珮慈來救我幫我開門；我不會忘記，為了趕 DATA，群超凌晨五點跟我一起做 Flow 實驗，實驗的生活有你們相挺而美好，祝你們往後的日子一切順利！

謝謝劉比奇在我心情低落的時候，大半夜願意傾聽我鬼哭神嚎，雖然目前我們的人生不如所願，但是我們的明天會更好！謝謝珮瑤、琳雅、志翔在我煩悶的時候，你們安排節目讓我難忘跟你們在台北瘋狂的夜晚！謝謝雅之、慧菁、銘富你們知道我心裡的苦，總是願意花時間陪伴我，就怕我一個人胡思亂想，那怕是駕車三十分鐘，只為了帶我離開台中到草屯吃一頓我沒胃口吃下的晚餐！我有你們，所以我很幸福！

謝謝 Samantha 和 Jerry 的婚禮，為了獻詩獻舞讓我在繁忙的實驗生活更加充實，也讓我相信這個世界有真正的愛情，我會和你們一樣有著屬於自己的幸福！謝謝國陽、煜鵬、永霖、思婷，在我遭遇挫折的時候，總是用上帝的話語鼓勵我為我禱告，相信我能勝過的！謝謝俞妙、靜枚乾媽，為我守望禱告！

謝謝我的家人，爸爸媽媽給我一個安全的避風港，即使在外受了多少的屈辱，家永遠是我的停靠的港口，謝謝當年遠在英國的哥哥總是會傳訊息要我撐住，謝謝妹妹在這一年的相伴，感謝上帝，我知道祢一直住在我心裡，一直陪伴著我，一直用最好的角度看待我，祢有最美妙的安排，謝謝祢讓我在這個看似困難的環境，卻給我滿滿的祝福！如今我要帶著祢給加給我的福分，勇闖人生另一個階段，我相信有祢無條件的愛，所以我的人生更加豐盛！謝謝所有關心我以及愛我的人，我也愛你們！

中文摘要

氧化型低密度脂蛋白(oxidized low-density lipoprotein；oxLDL)是動脈粥狀硬化前趨分子，它堆積在血管壁會造成血管壁的失能傷害。Lectin-like oxLDL receptor（LOX-1），是內皮細胞中oxLDL主要之接受器。已有研究指出LOX-1的活化可以刺激活性氧自由基（reactive oxygen species；ROS）的產生導致內皮細胞功能失調。銀杏萃取物(Ginkgo biloba extract；GbE)，萃取於銀杏樹的葉子，早被證實對於心血管疾病及神經系統有益處。然而有關其作用機制則有許多仍不清楚。本實驗主要探討銀杏萃取物保護oxLDL引起的內皮細胞傷害，是否經由調控LOX-1所媒介之訊息傳遞路徑。實驗方法為，利用oxLDL（130 μg/ml）誘發人類臍帶靜脈細胞內皮細胞傷害，以探討銀杏萃取物（12.5，25，50，100 μg/ml）之保護機制。實驗結果証明銀杏萃取物抑制由oxLDL所誘發內皮細胞LOX-1之基因及蛋白之表現，及oxLDL所誘發NADPH氧化酶（NADPH oxidase）之活化及其所產生之自由基，並抑制oxLDL所增加之誘發型一氧化氮合成酶（inducible nitric oxide synthase；iNOS）及其產生之一氧化氮與硝基酪氨酸（nitrotyrosine）。此外，oxLDL經由增加p38磷酸化，減少Akt、腺苷單磷酸活化蛋白質激酶（AMPK）-內皮細胞型一氧化氮合成酶（endothelial nitric oxide synthase；eNOS）磷酸化，而增加轉錄因子NF-κB（nuclear factor-κB）活化及其下游發炎相關基因之表現，如環氧化酶-2（cyclooxygenase-2；COX-2），第一型及第三型基質金屬蛋白酶（matrix metalloproteases-1 和 matrix metalloproteases-3；MMP-1 和 MMP-3）之表現，而銀杏萃取物前處理則可明顯

抑制上述oxLDL所誘發之氧化傷害。此實驗結果有助瞭解銀杏萃取物用於預防動脈

粥狀硬化等心血管疾病之分子機制。

Abstract

Background: Oxidized low-density lipoprotein (oxLDL) is a proatherogenic molecule that accumulates in the vascular wall and contributes to the pathogenesis of vascular dysfunction. LOX-1, a lectin-like oxLDL receptor, is responsible for binding and uptake of oxLDL in endothelial cells. It has been well documented that the activation of LOX-1 can stimulate the formation of ROS and initiate a cascade of redox-sensitive singnaling events. Ginkgo biloba extract (GbE), extracted from the leaves of the Ginkgo biloba tree, has been well known about its benefits in cardiovascular and neurological systems. In this study, we hypothesize that GbE protects against oxLDL-induced endothelial dysfunction by modulating the LOX-1-mediated signaling pathway. ***Methods:*** In this study, incubation of primary human umbilical vein endothelial cell culture (HUVECs) were pretreated with GbE 12.5, 25, 50, 100 μg/ml for 2 hours, and then incubated with oxLDL (130 μg/ml) for an additional 24 hours. ***Results:*** The results of this study showed that GbE or DPI (a well-known inhibitor of NADPH oxidase) reduced ROS production and up-regulation of LOX-1 caused by oxLDL. We also found that oxLDL increased action of $p47^{phox}$ and Rac-1, and the subsequent induction of ROS generation; nevertheless, ROS generation was significantly decreased in cells pretreated with GbE or anti-LOX-1 monoclonal antibody. Following, oxLDL up-regulated inducible NO synthase (iNOS),

thereby augmenting the formation of NO and protein nitrosylation. Furthermore, oxLDL also increased p38MAPK phosphorylation and decreased the phosphorylation of the Akt, AMPK, eNOS with maximal induction at about 30 min, and activated the NF-κB-mediated inflammatory, redox-sensitive signaling. Pretreatment with GbE; however, exerted significant cytoprotective effects in all events. ***Conclusion:*** These data suggest that GbE inhibits the oxLDL-induced LOX-1-mediated signaling pathway, at least in part, by inhibiting NADPH oxidase and consequent ROS-enhanced LOX-1 expression, which contributes to further ROS generation and the subsequent suppressing the release of NO by down-regulating eNOS and activation of NF-κB via the p38MAPK pathway. Results from this study may provide insight into a possible molecular mechanism by which GbE prevent oxLDL-induced endothelial dysfunction.

Keywords: Oxidized low density lipoprotein; Lectin-like ox-LDL receptor-1; Reactive oxygen species; endothelial cells; Ginkgo biloba extract

Index

1. 誌謝

2. 中文摘要(Chinese abstract)---i

3. 英文摘要(English abstract) ---iii

4. 引言(Introduction)--1

5. 材料與方法(Materials and methods) ---5

6. 結果(Results)---12

7. 討論(Discussion)---16

8. 圖表與圖表說明(Figures and Legends) ---20

Figure 1. Inhibitory effects of GbE (Ginkgo biloba extract) on oxLDL-induced endothelial LOX-1(a lectin-like oxLDL receptor-1) gene----------------20

Figure 2. Inhibitory effects of GbE on oxLDL-induced ROS generation in HUVECs.--21

Figure 3. Effects of GbE on oxLDL-induced p47phox and Rac-1 membrane translocation---22

Figure 4. Effects of GbE on oxLDL-up-regulated iNOS, nitrotyrosine protein expression and oxLDL-enhanced Nitrite accumulation.------------------23

Figure 5. Effects of GbE on phosphorylation of p38MAPK and ERK.------------24

Figure 6. Effects of GbE on activation of NF-κB.----------------------------------25

中國醫藥大學 CHINA MEDICAL UNIVERSITY

Figure 7. Effects of GbE on dephosphorylation of AMPK, Akt and eNOS.-------26

Figure 8. Effects of GbE on oxLDL-induced COX-2 expression through NF-κB activation.---27

Figure 9. Effects of GbE on oxLDL-induced endothelial MMP-1 and MMP-3 gene and protein expression through NF-κB activation .-----------------28

Figure 10. Effects of GbE on oxLDL-induced endothelial MCP-1 and CXCR-6 expression through NF-κB activation.--------------------------------------29

Legend 1. Inhibitory effects of GbE (Ginkgo biloba extract) on oxLDL-induced endothelial LOX-1(a lectin-like oxLDL receptor-1) gene----------------30

Legend 2. Inhibitory effects of GbE on oxLDL-induced ROS generation in HUVECs.--30

Legend 3. Effects of GbE on oxLDL-induced p47phox and Rac-1 membrane translocation--30

Legend 4. Effects of GbE on oxLDL-up-regulated iNOS, nitrotyrosine protein expression and oxLDL-enhanced Nitrite accumulation.------------------31

Legend 5. Effects of GbE on phosphorylation of p38MAPK and ERK.-----------31

Legend 6. Effects of GbE on activation of NF-κB.----------------------------------31

Legend 7. Effects of GbE on dephosphorylation of AMPK, Akt and eNOS.-----31

Legend 8. Effects of GbE on oxLDL-induced COX-2 expression through NF-κB

activation--32

Legend 9. Effects of GbE on oxLDL-induced endothelial MMP-1 and MMP-3 gene and protein expression through NF-κB activation .-----------------32

Legend 10. Effects of GbE on oxLDL-induced endothelial MCP-1 and CXCR-6 expression through NF-κB activation.-------------------------------------32

9. 參考文獻(References) ---35

Introduction

Atherosclerosis is a chronic inflammatory disease and result of the majority cardiovascular disease complications[1]. Atherogenesis involves modified low density lipoprotein (LDL) modifications which oxidized LDL, increase the uptake of plasma LDL into macrophages and give rise to foam cells. Oxidized low-density lipoprotein (oxLDL) leads to chronic inflammation, endothelial injury and oxidative stress[2]. Oxidative stress is caused by an overabundance of reactive oxygen species (ROS) or decline in antioxidant ability against them. Under pathological conditions, increased ROS activity leads to endothelial dysfunction, endothelial injury, increased contractility vascular smooth muscle cells (VSMC) growth, monocyte invasion, lipid peroxidation, inflammation, and increased deposition of extracellular matrix proteins, important factors in vascular damage[3]. Thus, oxidative stress is considered to play a key role in the pathogenesis of atherosclerosis and a strong inducer of endothelial LOX-1 expression[4]. The important initiating event for atherosclerosis may well be the transport of oxidized low-density lipoprotein (oxLDL) across the endothelium into the artery wall[1]. This increased attachment of cells is accompanied by extravasation of low-density lipoprotein (LDL) into the vessel wall, subsequent foam cell and plaque formation and, ultimately, cell necrosis[5]. Disruption to structures in adherens membrane junction that damaged endothelial cells, results in more and more increased passage of pro-atherosclerotic compounds into the vessel wall. This series of adverse changes is also associated with a decrease in nitric oxide (NO) activity that results in a reduced ability of the endothelium to control vessel tone[6].

The enzymatic sources of superoxide anion include xanthine oxidase, myeloperoxidase, lipoxygenase, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. NADPH oxidase has five subunits: $p47^{phox}$ (“phox” stands for

phagocyte oxidase), $p67^{phox}$, $p40^{phox}$, $p22^{phox}$, and the catalytic subunit $gp91^{phox}$ (also termed “Nox2”)[7]. On stimulation, $p47^{phox}$ becomes phosphorylated and the cytosolic subunits form a complex that translocates to the membrane, where it associates with gp91 and $p22^{phox}$ to assemble the active oxidase, which transfers electrons from the substrate to O_2, forming $O_2.^{-}$ [8]. Activation also requires participation of Rac-1[9]. OxLDL, via LOX-1 receptoes, is an important and potent regulator of cardiovascular NADPH oxidase that activates NADPH oxidase through stimulation of signaling pathways involving P38 mitogen-activated protein kinase (P38MAPK) and phosphatidylinositol-3-kinase (PI3K) [10], both causing NF-κB activation. Additionally, oxLDL facilitates endothelial cell apoptosis and inhibits angiogenesis while promoting activation of endothelial NF-κB[11]. NF-κB, a key transcription factor is directly activated by ROS[12], which also controls the expression of a number of pro-inflammatory molecules, including adhesion molecules, interleukins[13], cyclooxygenase-2 (COX-2)[14] and in macrophage-derived matrix metalloproteases-1 and matrix metalloproteases -3 (MMP-1 and MMP-3) secretion[15], that play a role in vascular inflammation associated with atherosclerosis. Previous studies had also revealed that oxLDL activates NF-κB at least in part through p38 MAPK and PI3K transduction pathway[16].

LOX-1, lectin-like oxidized low density lipoprotein receptor-1, the new kind of scavenger receptor expressed in ECs, SMCs and macrophages[17]. LOX-1 was identified the main receptor for oxLDL responsible for binding, internalization, and degradation of oxLDL in endothelial cells[2] and it induces endothelial dysfunction triggered by oxLDL[18]. Thus, it was believed an important role in the pathogenesis of atherosclerosis[16] cardiovascular diseases. Many previous studies showed oxLDL binds to LOX-1 induced ROS production and results in activation of NF-κB. It also

been reported in several cardiovascular diseases that oxLDL enhanced LOX-1 mRNA and protein expression. The signal transduction pathways of LOX-1 could be mediated by oxLDL induced the increased production of intracellular ROS via NADPH oxidase activation, both of them are products of LOX-1 activation and inducers of LOX-1 expression. The downstream changes activation of NADPH oxidase[19] and subsequent redox signals involving p38MAPK, PI3K, which led to activation of the transcription factor NF-κB[20]. These steps play a crucial role in the subsequent cell injury, including the translocation of the transcription factor NF-κB and generation of ROS and secretion of pro-atherogenic enzymes [e.g. MMPs][21]. The result of ROS accumulates in endothelial space activated NF-κB-mediated pathway [22] and influenced PI3K, P38 MAPK expression and decrease NO release[11, 23]. ROS plays an important role in oxLDL-induced apoptosis and NADPH oxidase also involved in oxLDL-induced apoptosis.

Matrix metalloproteinases (MMPs) regulate the behavior of vascular wall cells in different atherosclerosis stages[24]. OxLDL induced monocytes and macrophages express in atherosclerotic lesions express matrix metalloproteinases including MMP-1, MMP-3, and MMP-9, has been demonstrated in human atherosclerotic plaques and in animal models[25]. They degrade the collagen and other extracellular matrix proteins, are involved in the accelerated breakdown of the extracellular matrix associated with vascular remodeling during the development of atherosclerosis [26], thus weakening atherosclerotic plaques and increasing the plaque rupture[27]. Lesions showed regionally increased MMP expression. In pathological conditions associated with local release of cytokines in the vessel wall, enhanced regional expression of vascular MMPs may contribute to SMC migration and weakening of matrix that would favor plaque rupture, events associated with the development or complication of the

atherosclerotic lesions[27]. It is evident that the metabolic balance of extracellular matrix is regulated in large part by matrix metalloproteinases[24].

Ginkgo biloba extract (GbE), extracted from the leaves of the Ginkgo biloba tree , is a defined complex mixture containing 24% ginkgo flavone glycoside and 6% terpenlactones (ginkgolides, bilobalide)[28]. GbE have been found to possess antioxidantion[29], antitumor[30], antiaging [31], antiatherosclerosis [32]and cardioprotective properties[33]. GbE exhibits beneficial effects on preventing oxidative stress associated with this process[34]and the pharmacological effects of GbE are closely related to its antioxidant ability to scavenge free radicals[35] [36] [37]. In addition, GbE also inhibits smooth muscle cell proliferation, vascular endothelial growth factor[38], expression of adhesion molecules and prevents oxLDL-induced apoptosis in endothelial cells[29]. However, to our best knowledge, there was no study show GbE could against oxLDL bind to LOX-1 and NADPH oxidase activity.

The aim of this study was to examine the effect of GbE could protects against oxLDL-induced endothelial dysfunction through LOX-1-mediated signaling pathway. We undertook the current study to explore the effects of GbE on oxLDL-induced ROS generation, NADPH oxidase activation, the translation of NF-κB after exposure to oxLDL. In addition, LOX-1 expression and and NF-κB-relative downstream inflammatory responses to HUVECs were also determined.

Materials and Methods

Reagents. Fetal bovine serum (FBS), medium 199(M199) and trypsin-EDTA were obtained from GIBCO (Grand Island, NY, USA); low serum growth supplement were obtained from Cascade (Cascade Biologics, Portland, OR). GbE, a defined complex mixture containing 24% ginkgo flavones glycoside (primarily composed of quercetin, kaempferol, and isorhamnetin) and 6% terpenlactones (ginkgolides A, B, and C and bilobalide) extracted from Ginkgo biloba leaves, was obtained from Dr. Willmar Schwabe (Karlsruhe, Germany); diphenyleneiodonium (DPI), penicillin (Sigma, MO, USA); anti-LOX-1, anti-MMP-1 (R&D Systems, MN, USA); anti-NF-κB/p65, anti-IκB (Transduction Laboratories, CA, USA). anti-Rac-1 and anti-p47^{phox} (BD Biosciences, NJ, USA); anti-cyclooxygenase-2 (COX-2) (Abcam, MA, USA). anti-gp91 and anti-Nitrotyrosine(Santa Cruz, CA, USA), anti-MMP-3(Gene Tex, CA, USA), anti-p38MAPK, anti-Akt, anti-AMPK, anti-eNOS(Cell signaling, MA, USA), anti-PCNA(Millipore, MA, USA)

Cell cultures.

This experiment was approved by the Research Ethics Committee of China Medical University Hospital. After receiving written informed consent from the parents, fresh human umbilical cords were obtained from neonates after birth, collected and suspended in Hanks' Balanced Salt Solution (HBSS) (Gibco, USA) at 4℃. Human umbilical vein endothelial cells (HUVECs) were isolated with collagenase and used at passage 2-3 as previously described[39]. After dissociation, the cells were collected and cultured on gelatin-coated culture dishes in medium 199 with low serum growth supplement, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Subcultures were performed with trypsin-EDTA. Media were refreshed on every two days. The

identity of umbilical vein endothelial cells was confirmed by the appearance of a cobblestone morphology and a strong positive immunoreactivity to von Willebrand factor.

Lipoprotein separation.

Human plasma was obtained from the Taichung BloodBank (Taichung, Taiwan) and LDL was isolated using sequential ultracentrifugation(=1.019-1.210 g/ml) in KBr solution containing 30 mM EDTA, stored at 4℃ in sterile, dark environment and used within 3 days as previously described [40]. Immediately before the oxidation tests, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 Sephadex G-25 Mgel (Pharmacia) in 0.01 mol/l phosphate-buffered saline (136.9 mmol/l NaCl, 2.68 mmol/l KCl, 4 mmol/l Na2HPO4, 1.76 mmol/l KH2PO4) at pH 7.4. Cu^{2+}-modified LDL (1mg protein/ml) was prepared by exposing LDL to 10 μM $CuSO_4$ for 16 hours at 37℃. Protein was measured by the method used by Bradford [41].

Isolation of mRNA and real-time PCR.

Total RNA was isolated from the HUVECs using the RNeasy Kit (Qiagen, Valencia, CA). Oligonucleotides (LOX-1, MMP-1, MMP3 and GADPH) were designed using the computer software package Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA). All oligonucleotides were synthesized by Invitrogen (Invitrogen, Breda, Netherlands). The sequences of gene forward (F-) and reverse (R-) primers are as follows:LOX-1(F-):CAGAGTTCGACACTACGTCA,(R-):GATGCCCCACTTGTTCAGAT;COX-2(F-):TGAGCATCTACGGTTTGCTG,(R-)AACTGCTCATCACCCCATTC;MMP-1(F-):TGGGATATTGGAGCAAGAGGCT,(R-)GCAGCAGCAGCAGTGG

AGGA.MMP-3(F-):ACCTGACTCGGTTCCGCCTGT,(R-)CAGTTGGCTGGCGTC CCAGG. Oligonucleotide specificity was computer tested (BLAST, National Center for Biotechnology Information) by homology search with the human genome and later confirmed by dissociation curve analysis. PCRs were performed using the SYBR Green method in an ABI 7000 sequence detection system (Applied Biosystems) according to the manufacturer's guidlines .The reactions were set by mixing 12.5 μl of the SYBR Green Master Mix (Applied Biosystems, Foster city, CA, USA) with 1 μl of a solution containing 5 nmol/μl of both oligonucleotide ,and 1 μl of a cDNA solution(1/100 of the cDNA synthesis product). The cycle threshold (Ct) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (fixed at 0.2 relative fluorescence units). This threshold was set constant throughout the study and corresponded to the log linear range of the amplification curve.

Measurement of ROS production.

The effect of GbE on ROS production in HUVECs was determined by a fluorometric assay using by 2',7'-dichlorofluorescein acetoxymethyl ester (DCF-AM) as a probe for the presence of superoxide. Confluent HUVECs (10^4 cells/well) in 96-well plates were preincubated with various concentrations of GbE for 2 hours; oxLDL was then added to the medium in the absence or presence of GbE for 2 hours. After the removal of medium from wells, cells were incubated with 10 μM DCF-AM for 1 h. The fluorescence intensity was measured with a fluorescence microplate reader (Labsystem, CA) calibtated for exciation at 485 nm and emission at 538 nm. The percentage increase in fluorescence per well was calculated by the formula $[(Ft_2-Ft_0)/Ft_0]$ X 100, where Ft_2 is the fluorescence at 2 hours of oxLDL exposure and

Ft_0 is the fluorescence at 0 min of oxLDL exposure. OxLDL-induced superoxide formation, which occurs largely through activation of NADPH oxidase, but also through uncoupling of endothelial NO synthase, xanthine oxidase, peroxisomes, and through direct superoxide release, leads to endothelial dysfunction. In this regard, diphenyleneiodonium (DPI) , a potent inhibitor of flavin-containing oxidase, has frequently been used to inhibit ROS production mediated by various flavoenzymes, including NADPH oxidase, quinine oxidoreductase, cytochrome P450 reductase and nitric oxide synthase[42]. To evaluate the role of NADPH oxidase and LOX-1 in oxLDL-induced ROS generation, cells were preincubated with DPI (5 μM) and anti-LOX-1 mAb (40 μg/ml) for 2 hours before exposure to oxLDL.

Nitrite (NO_2^-) accumulation

NO_2^- accumulation was used as an indicator of NO production in the medium and was assayed by Gries reagent[43]. Briefly, 100 μl of Gries reagent (1% sulfanilamide–0.1% naphthylethylene diamine dihydrochloride–2.5% H3PO4) (Sigma, St. 12 Louis, MO) was added to 100 μl of each supernatant in triplicate wells of 96-well plates. The plates were read in a microplate reader (Molecular Devices, Palo Alto, CA, USA) at 550 nm against a standard curve of $NaNO_2$ in culture medium.

Immunoblotting.

To determine whether GbE could ameliorate the oxLDL-induced LOX-1-regulating protein HUVECs were grown to confluence, pretreated with GbE for 2 h and then stimulated with oxLDL for 24 h. At the end of stimulation, cells were washed, scraped from dishes, and lysed in RIPA buffer (in mM: HEPES 20, MgCl2 1.5, EDTA 2, EGTA 5, dithiothreitol 0.1, phenylmethylsulfonyl fluoride 0.1, pH 7.5). Proteins (30

μg) were separated by electrophoresis on SDS-polyacrylamide gel. Cytosolic p38, p-p38, Akt, p-Akt, AMPK, p-AMPK, eNOS, p-eNOS, LOX-1, COX-2, IκB, and membrane p47phox and Rac-1, MMP-1,3, iNOS, nitrotyrosine as well as nucleic NF-κBp65 were determined by SDS-PAGE and immunoblot assay. The blots was incubated with blocking buffer (1X PBS and 5% nonfat dry milk) for 1 hour at room temperature and then probed with primary antibodies (1:1000 dilutions) overnight at 4℃ , followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour. To control equal loading of total protein in all lanes, blots were stained with mouse anti-actin antibody at a 1:50000 dilution. The bound immunoproteins were detected by an enhancer chemiluminescent assay (ECL; Amersham, Berkshire, UK). The intensities were quantified by densitometric analysis (Digital Protein DNA Imagineware, Huntington Station, NY).

Nuclear protein extraction.

Cells grown to 80% confluency and subjected to various treatments will be subsequently washed with ice-cold PBS and it's will be prepared for nuclear protein extraction. Cells grown on 10-cm dish will be gently scraped with 3 ml ice-cold PBS and it's will be centrifuged at 1,000g for 10 min at 4°C. After carefully aspirating the supernatant, cells will be resuspended with 200 μl ice-cold BUFFER-I (10 mM Hepes (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, and proteinase inhibitor cocktail (Roche Molecular Biochemicals) and its will be incubated for 15 min on ice to allow cells to swell, followed by adding 20 ll IGEPAL-CA630. After vigorously vortexing for 10 s and centrifuging at 16,000 g for 5 min at 4℃, the supernatant (cytoplasmic fraction) will be carefully aspirated and the pellet will be resuspended with ice-cold BUFFER-II (20 mM Hepes (pH 8.0), 1.5 mM MgCl2, 25% glycerol,

420 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol and proteinase inhibitor cocktail (Roche Molecular Biochemicals)) and vigorously vortex. After vortexing, the suspension will be placed on ice for 30 min before centrifuging at 16,000g for 15 min at 4℃. The supernatants (nuclear extracts) will be stored aliquots at -80℃. Protein concentration of the supernatants will be determined by the colorimetric assay (Bradford).

Membrane protein extraction.

We used Mem-PER® Eukaryotic Membrane Protein Extraction Reagent Kit (PIERCE, 89826) to extract membrane protein. Cells grown to 80% confluency and subjected to various treatments were subsequently washed with ice-cold PBS and it was prepared for membrane protein extraction. Cells grown on 10-cm dish gently scraped with 3 ml ice-cold PBS and it was centrifuged at 10,000g for 10 min at 4℃. After carefully aspirating the supernatant, cells added 150 μl of Reagent A to the cell pellet. Pipette up and down to obtain a homogeneous cell suspension. Incubate 10 minutes at room temperature with occasional vortexing, then added 450 μl of diluted Reagent C to each tube of lysed cells and vortex. Incubate tubes on ice for 30 minutes, vortexing every 5 minutes. Centrifuge tubes at 10,000 g for 3 minutes at 4℃. Transfer supernatant to new tubes and incubate 10 minutes in 37℃ water bath to separate the membrane protein fraction. Centrifuge tubes at room temperature for 2 minutes at 10,000 g to isolate the hydrophobic fraction (i.e., the fraction containing membrane protein of interest) from the hydrophilic fraction, Carefully remove the hydrophilic phase (top layer) from the hydrophobic protein phase (bottom layer) and save in a new tube. Perform phase separations as quickly as possible because the interface between the layers slowly disappears at room temperature. Place the separated

fractions on ice. The supernatants (membrane protein extracts) will be stored aliquots at -80℃. Protein concentration of the supernatants will be determined by the colorimetric assay (Bradford).

Adhesion molecule expression

To determine whether GbE could modify the oxLDL-induced adhesion molecule expression, HUVECs were grown to confluence, pretreated with GbE for 2 hours and stimulated with oxLDL (130 μ g/ml) for 24 hours. At the end of stimulation, HUVECs were harvested and incubated with FITC-conjugated anti-CXCR6 and anti-MCP-1 (R&D, Minneapolis, MN) for 45 min at room temperature. After the HUVECs had been washed three times, their immunofluorescence intensity was analyzed by flow cytometry using a Becton Dickinson FACScan flow cytometer (Mountain View, CA, USA).

Statistical analyses. All experiments were repeated 3 times, and one of these results is provided. Results are expressed as mean±SEM. Differences between the groups were analyzed using one-way ANOVA followed by the Student's t test. A P-value <0.05 was considered statistically significant.

Results

GbE inhibited oxLDL-induced LOX-1 gene and protein expression

The inhibitory effects of GbE on LOX-1 mRNA and protein were analyzed by real-time PCR and Western blot , respectively. The levels of LOX-1 mRNA (Fig.1A) and protein (Fig.1B) expression in HUVECs were enhanced by oxLDL (130 μg/ml). Treatment of HUVECs with GbE for 2 hours at concentrations above 12.5 μg/ml before exposure to oxLDL for 24 hours resulted in suppression of LOX-1 expression both in mRNA and protein levels. In addition, pretreatment with DPI, an inhibitor of ROS production, markedly inhibited oxLDL-induced LOX-1 up-regulation, strongly suggesting that ROS plays a critical role in the increased protein expression of LOX-1.

GbE inhibited oxLDL-induced ROS generation in HUVECs.

The effect of GbE on the LOX-1-mediated redox-sensitive signaling pathway was analyzed by fluorescence microscopy. The level of ROS generation has significantly decreased in endothelial cells pretreated with GbE(12.5-100 μg/ml) for 2 hours followed by exposure to oxLDL(130 μg/ml) in a dose-dependent manner (all $P<0.05$) (Fig. 2A and 2B). Additionally, the level of ROS induced by oxLDL was abolished by pretreatment with monoclonal antibody of LOX-1 (mAb anti-LOX-1) or the diphenyleneiodonium inhibitor DPI (Fig. 2B), suggesting that oxLDL induced ROS production and the mechanism is dependent on binding of oxLDL to LOX-1 and subsequent activation of NADPH oxidase.

Effects of GbE on oxLDL-induced membrane translocation of $p47^{phox}$ and Rac-1.

The effects of GbE on membrane translocation of $p47^{phox}$ and Rac-1 were examined

by Western blotting in HUVECs with membranes and cytosolic isolation. Membrane translocation assay showed that the levels of $p47^{phox}$ and Rac-1 in membrane fractions of HUVECs were 4~5-fold higher in cells treated with oxLDL for 30 min than those in untreated cells (Fig. 3A-C). The enhanced $p47^{phox}$ and Rac-1 on membrane translocation by oxLDL were inhibited by pretreatment with GbE in a dose-dependent manner as well as they were also inhibited by NADPH oxidase inhibitor DPI. In addition, we found that the protein levels of gp91 and $p22^{phox}$ were increased significantly in HUVECs exposed to oxLDL for 24 hours. However, pretreatment of oxLDL-exposed cells with GbE (12.5-100 μg/ml) led to a dose-dependent reduction in gp91 protein expression (Fig. 3D)as well as they were also inhibited by NADPH oxidase inhibitor DPI.

Effects of GbE on oxLDL-induced iNOS , nitrotyrosine protein expression and nitrite (NO_2^-) accumulation

It is well known that oxLDL-induced endothelial dysfunction is associated with up-regulation of iNOS which is an important component in the systemic inflammatory respones. ROS, especially superoxide, generated by oxLDL directly reacts with NO to form peroxynitrite, a stable molecule that is toxic to endothelial cells. Therefore, to determine the effects of GbE on protein expression of NOS as well as NO content and formation of nitrotyrosine, a stable final metabolite of peroxynitrate that can serve as a fingerprint for peroxynitrite-mediated damage of cellular proteins after exposure to oxLDL. As shown in fig 4, oxLDL enhanced iNOS protein expression levels returned to levels close to those seen in control cells when HUVECs were treated with 100 μg/ml GbE prior to stimulation with oxLDL. Furthermore, the oxLDL-enhanced nitrosylation of tyrosine and nitrite (NO_2^-) accumulation were suppressed in HUVECs

pretreated with GbE as well as they were also inhibited by iNOS-specific inhibitor 1400W.

Effects of GbE on oxLDL-induced p38MAPK and ERK phosphorylation.

OxLDL-induced ROS can activate two signal transduction pathways involving either p38MAPK, ERK or Akt, both causing NF-κB activation, which facilitates nuclear translocation and subsequent regulation of pro-inflammatory gene expression [20]. The key step in NF-κBp65 nuclear translocation is mediated by degradation of cytosolic IκB-α. Thus, we used immunoblotting to examine the effects of GbE on protein levels of NF-κBp65 and IκB-α in nuclear and cytosol extracts. As shown in Fig. 5A-C, incubation of HUVECs with oxLDL resulted in significant phophorylation of p38MAPK and ERK within 30 min without affecting their protein levels; this tendency was reversed significantly with GbE pretreatment. As shown in Fig. 6A-C, HUVECs exposed to oxLDL (130 μg/ml, 1 hour) the protein levels of NF-κBp65 in the nuclear fraction increased and the levels of IκB-α in cytosol decreased thereby causing nuclear translocation of NF-κBp65. In contrast, the HUVECs pretreated with GbE (12.5-100 μg/ml), the nuclear translocation of NF-κBp65 protein decreased in a dose-dependent manner ($P < 0.05$).

GbE ameliorated oxLDL-induced dephosphorylation of AMPK, Akt and eNOS.

Nitric oxide (NO) is produced in endothelial cells by a constitutively expressed enzyme known as eNOS which represents an important signaling molecule downstream of AMPK and Akt. Therefore, we performed western blot analyses to determine the effect of GbE treatment on this pathway. As shown in Fig. 6A-C, incubation of HUVECs with oxLDL resulted in significant dephosphorylation of AMPK, Akt and eNOS (a downstream effector of PI3K)(Fig.7 A-D) within 30 min without affecting their protein levels; this tendency was reversed significantly with

GbE pretreatment.

GbE supressed oxLDL-induced NF-κB-related inflammatory cytokines.

Degradation of IκB liberates NF-κB, which is then transferred from the cytoplasm to the nucleus where it activates the transcription of various inflammatory cytokines, genes encoding COX-2, MMP-1,3 and cell adhesion molecules MCP-1 and CXCR6 . Therefore, we sought to determine whether GbE inhibits oxLDL-induced expression of COX-2(Fig.8) and other inflammatory molecules. We found that MMP-1, MMP-3 and COX-2 gene expression (Fig.9A) and MMP-1, MMP-3, COX-2 protein expression (Fig.9B-D) were increased in cells exposed to oxLDL. As expected, pretreatment with GbE significantly inhibited both MMP-1, MMP-3 and COX-2 gene expression and MMP-1, MMP-3, COX-2 protein expression in a dose-dependent manner (all $P<0.05$). In addition, as shown as Fig.10, the expression levels of MCP-1 and CXCR6 were significantly higher in HUVECs that had been treated with oxLDL (130 μg/ml) for 24 h than in the control cells. Flow cytometry revealed that the induction of adhesion molecule expression was significantly ameliorated by the presence of 12.5-100 μg/ml GbE (all $P<0.05$).

Discussion

It is well known that oxLDL is a pro-atherogenic molecule that plays an important role in atherosclerosis. LOX-1 is the specific receptor for oxLDL highly expressed in endothelial cells, vascular smooth muscle cells and macrophages, it was believed an important role in the pathogenesis of atherosclerosis[19]. OxLDL binds to LOX-1 activates NADPH oxidase on the cell membrane and quick increase intracellular ROS generation, overabundance ROS accumulates in subendothelial space up-regulated LOX-1 expression. The consequent formation of ROS may be the first event in the chain of reactions leading to NF-κB activation contribute to further ROS generation[22]. This thesis is the first to show the effectiveness of GbE in suppressing endothelial LOX-1 expression and LOX-1 mediated pro-atherogenic effects. The effect of GbE on endothelial LOX-1 expression appears to be exerted at the transcriptional level, as reflected by the parallel decrease in LOX-1 mRNA and protein levels in GbE-treated cells (Fig 1). Moreover, pretreatment with DPI or blockade of LOX-1 with anti-LOX-1 mAb prevented oxLDL-induced ROS generation, which suggests that the binding of oxLDL to LOX-1 and the consequent formation of ROS may be the first event in LOX-1 mediated endothelial dysfunction. OxLDL regulates LOX-1 gene expression via redox-sensitive pathway[44] and involves NF-κB[45] which related pro-inflammatory molecules expression[14]. In this study demonstrated that GbE decreased ROS generation (Fig 2), NF-κB activation, and LOX-1 gene and protein expression supports this possibility.

NADPH oxidase is the major source of ROS in vascular tissues[46]. We measured the expression of NADPH oxidase and nitrotyrosine(oxidant stress marker), and found that the expressions of NADPH oxidase ($gp91^{phox}$, $p47^{phox}$ subunits and Rac-1) and nitrotyrosine were increased dramatically in oxLDL treated HUVECs. This increase

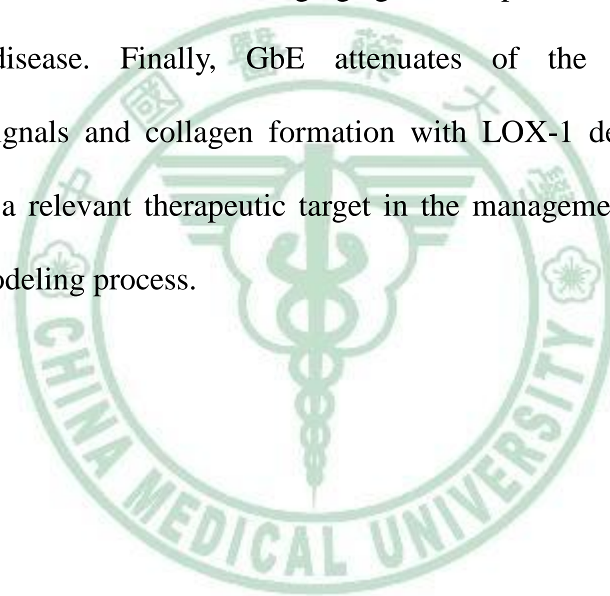
in NADPH oxidase and nitrotyrosine was much less in pretreated with GbE suggests that the beneficial effects of GbE may be due to suppression of the assembly of NADPH oxidase enzyme complex (Fig 3,4). In agreement with the study of Li et.al showing a role for LOX-1 in oxLDL induced MMP-1 and MMP-3 expression in human coronary endothelial cells[47]. Further, a couple of previous study suggest a strong link between NADPH oxidase-induced oxidant stress and MMPs expression. In keeping these study, we found out that GbE attenuated the expression of MMPs and had low levels of NADPH oxidase (gp91^{phox}, p47^{phox} subunits and Rac-1) dephosphorylation of Akt (a downstream effector of PI3K) (Fig 3). We demonstrated that GbE prevented ROS generation and induction of endothelial MMP-1 and -3 expression by oxLDL. LOX-1 activation has also been shown to activate NADPH oxidase and subsequent redox signals involving p38MAPK and NF-κB, the latter of which controls the expression of a number of cytokines, chemokines, inflammatory enzymes, and adhesion molecules in endothelial cells [19].

Atherosclerotic regions have reduced activity of eNOS and reduction of locally released NO may enhance oxidative stress and cell proliferation[2]. In contrast, the level of NO produced by inducible NO synthase (iNOS) is several orders of magnitude higher than that produced by eNOS in atherosclerotic lesions. In addition, the increase in NADPH oxidase activity leads to eNOS uncoupling, which results in the generation of superoxide rather than NO and it plays an important role in regulation of vascular tone. In the present study showed nitrite formation was largely parallel to the expression level of iNOS (Fig 4A, 4B) suggest that generation of NO as a precursor of nitrite in oxLDL-treated cells is due mainly to iNOS. However, pretreatment of HUVECs with GbE suppressed the oxLDL-induced up-reguation of iNOS, thereby leading to a reduction in protein nitrosylation (Fig. 4A, 4C) and NO production (Fig. 4D) as well as treated with iNOS inhibitor 1400W. PI3K/Akt has been identified as a pro-survival factor and a critical

signal transduction pathway in oxLDL/LOX-1-induced apoptosis in human vascular smooth muscle cells [23,24]. Akt is important in downstream targeting of extracellular PI3 kinase signaling, and alterations in its activity may be important in phosphorylation of NOS in response to oxidant stimuli[48]. In keeping with this concept, we observed a reduction of Akt phosphorylation and diminished expression eNOS in treated with oxLDL; however, the phenomenon was returned in pretreated with GbE. Previous studies have also documented that LOX-1 is key to altered endothelium-dependent vasorelaxation in atherosclerosis[49]. In addition, NO released from eNOS inhibits cleavage of IκB and NF-κB activation, which is enhanced by ROS[50]. Consistent with a previous study, our data showed that the antiathrogenic effects of GbE are due, at least in part, to the inhibition of adhesion molecules and subsequent monocytic adherence by moderating the reduction in eNOS expression caused by oxLDL. Our results showed that the protective effects of GbE inhibited NADPH and abolished on oxLDL-induced endothelial cell result in further stress .

The concentrations of GbE(12.5-100 μg/ml) required to suppress the oxLDL-induced endothelial dysfunction in our study were similar to those reported to inhibit other responses, such as smooth muscle cell proliferation, vascular endothelial growth factor, thrombomodulin expression, and tissue-type plasminogen activator secretion [38,51]. The dose of GbE used in previous in vitro studies, which usually ranged from 200 to 400 μg/ml, seems to be relatively high compared with the dose used in the current study. The recommended dose of GbE injection is 87.5 mg/day for patients with chronic vascular disease[52]. In humans, it is unclear how much the circulating blood level would be elevated by a single dose of GbE, since the pharmacokinetics of the components of GbE have not been completely established. It also is unknown whether prolonged use of GbE would lead to chronic accumulation of some of the components in different tissues.

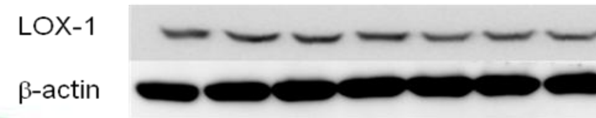
In summary, the present results demonstrated that GbE could prevent oxLDL-induced ROS and multiple mechanisms for ROS signal transduction in ox-LDL-induced NADPH oxidase activity and LOX-1 might be involved with endothelial dysfunction. Because this is a purely in vitro work which presents some limitations in regard to the diversity of endothelial oxLDL receptors, further studies are required to confirm the extent of contribution of oxLDL–LOX-1 interaction in terms of GbE effect on the inhibition of oxLDL mediated pro-atherogenic effects and the effectiveness of this drug in vivo. If similar effect is observed in vivo, GbE might imply that antioxidants or radical-scavenging agents has potential use in prevention of atherosclerosis disease. Finally, GbE attenuates of the expression of the redox-sensitive signals and collagen formation with LOX-1 deletion suggests that LOX-1 could be a relevant therapeutic target in the management of atherosclerosis and vascular remodeling process.



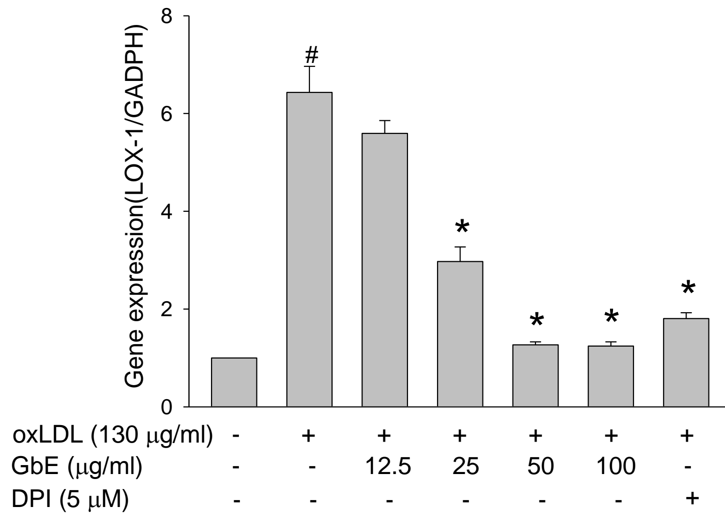
Figures

(B)

oxLDL (130 μg/ml)	-	+	+	+	+	+	+
GbE (μg/ml)	-	-	12.5	25	50	100	-
DPI (5 μM)	-	-	-	-	-	-	+



(A)



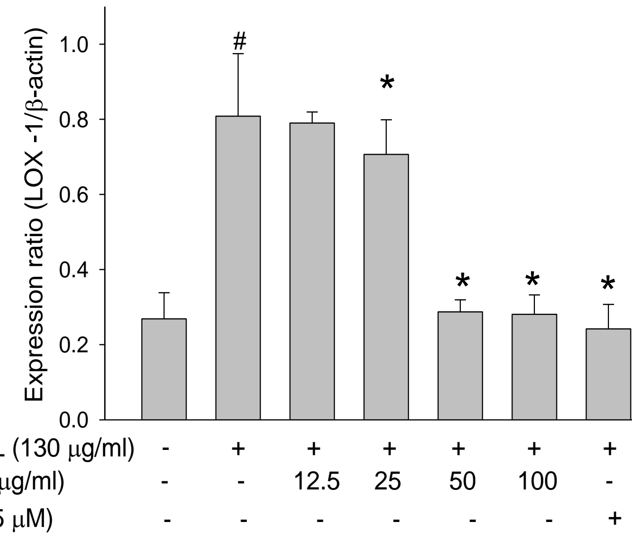
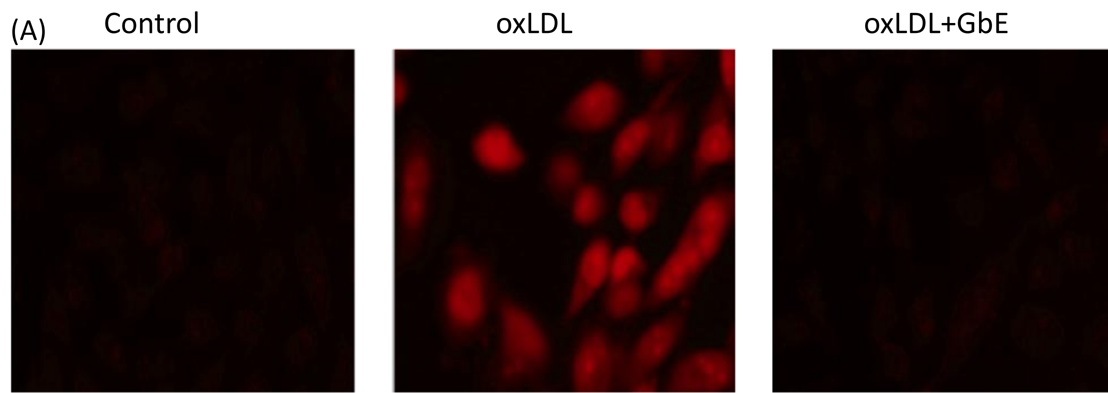


Fig.1 Inhibitory effects of GbE (Ginkgo biloba extract) on oxLDL-induced endothelial LOX-1(a lectin-like oxLDL receptor-1) gene



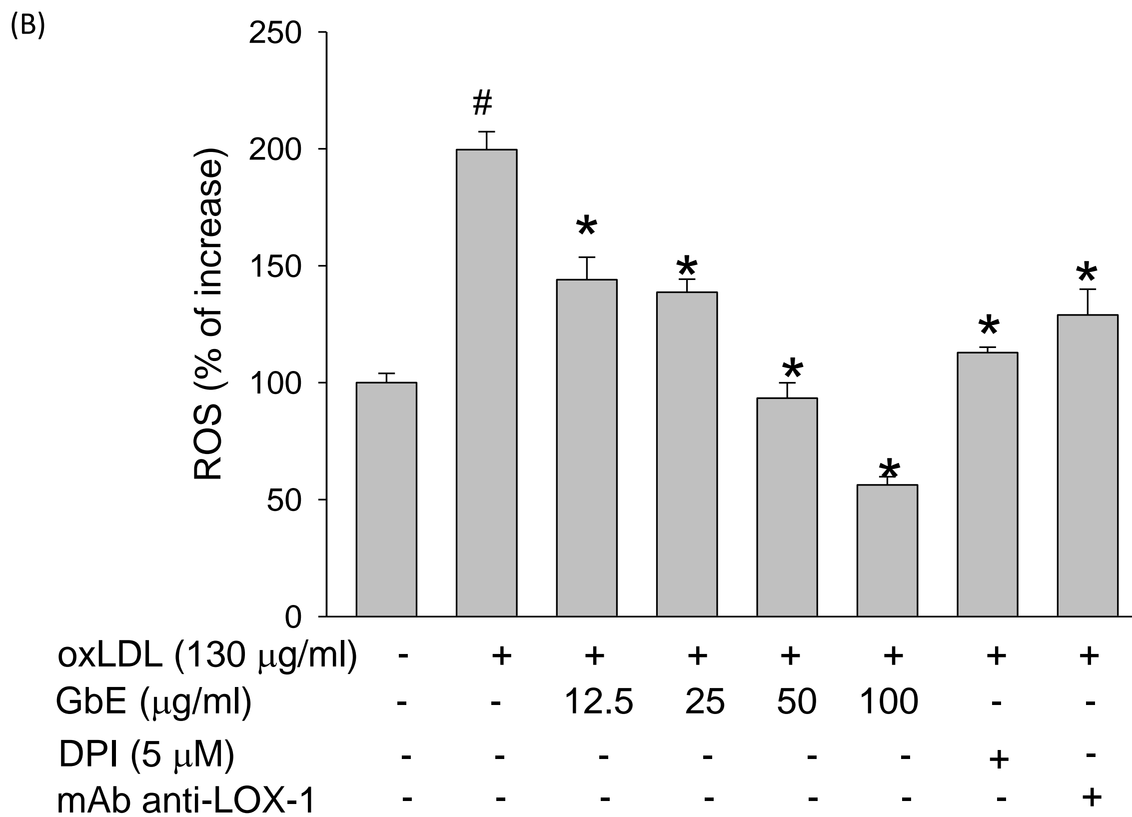


Fig.2 Inhibitory effects of GbE on oxLDL-induced ROS generation in HUVECs

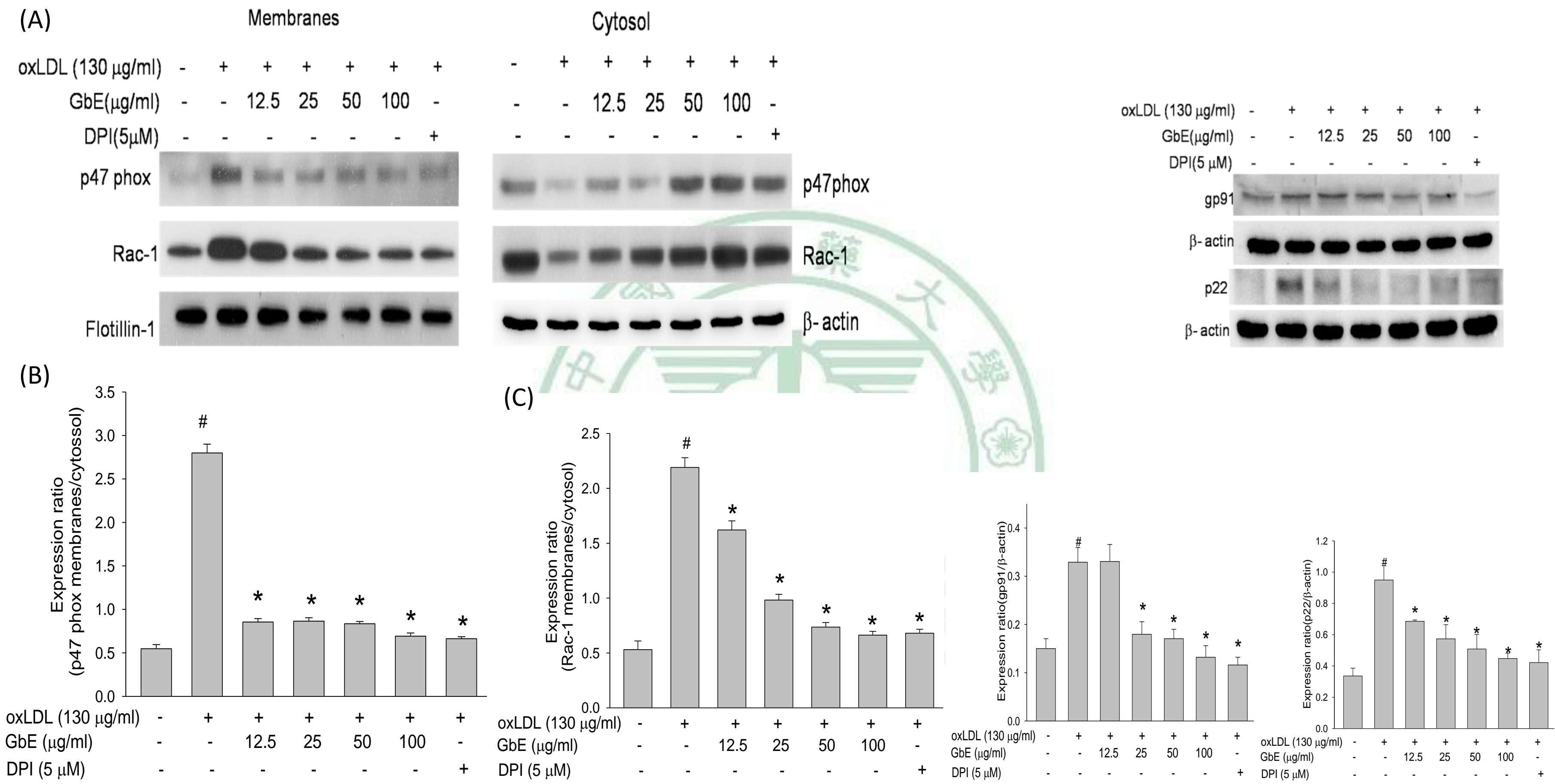
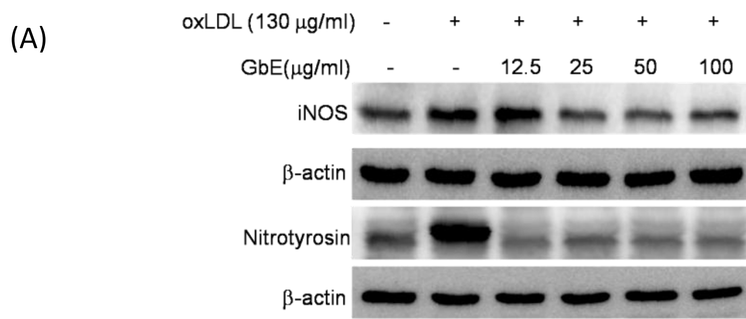
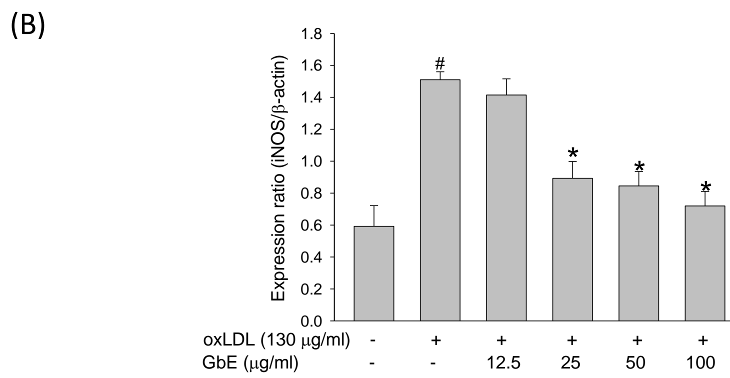


Fig.3 Effects of GbE on oxLDL-induced p47phox and Rac-1 membrane translocation

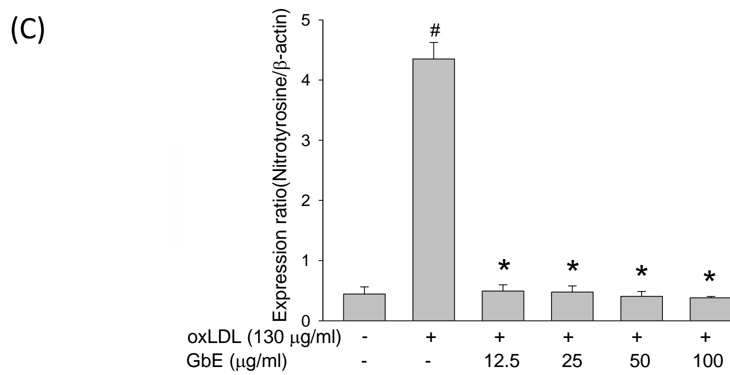
(A)



(B)



(C)



(D)

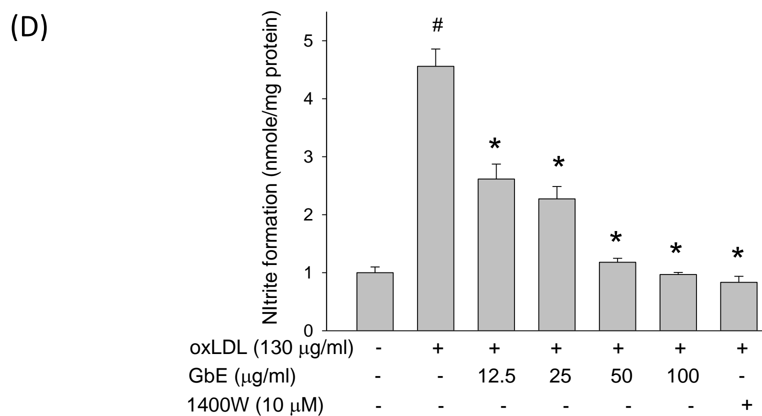
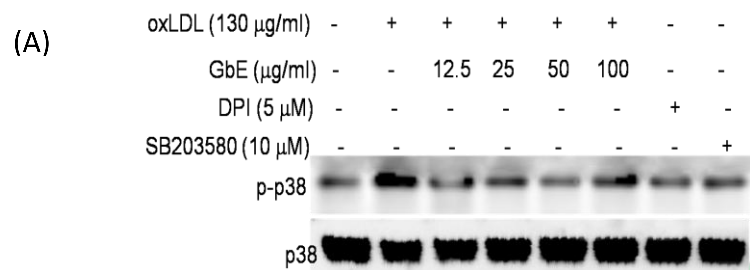
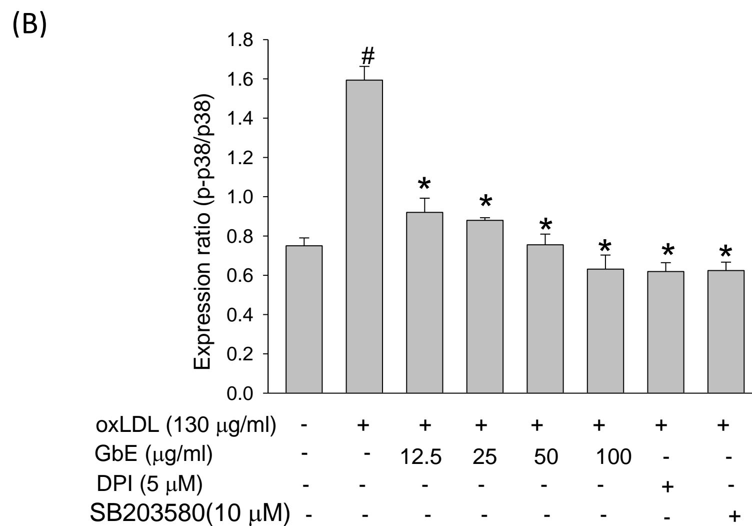
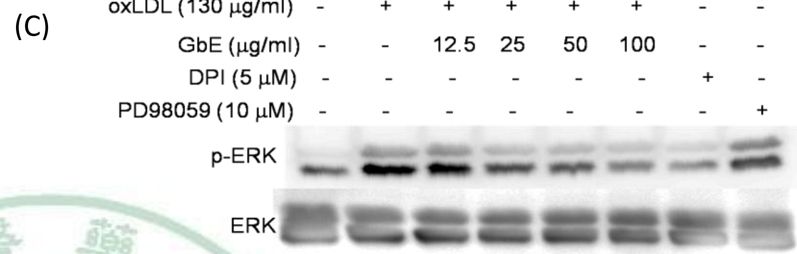


Fig.4 Effects of GbE on oxLDL-up-regulated iNOS, nitrotyrosine protein expression and oxLDL-enhanced Nitrite accumulation.







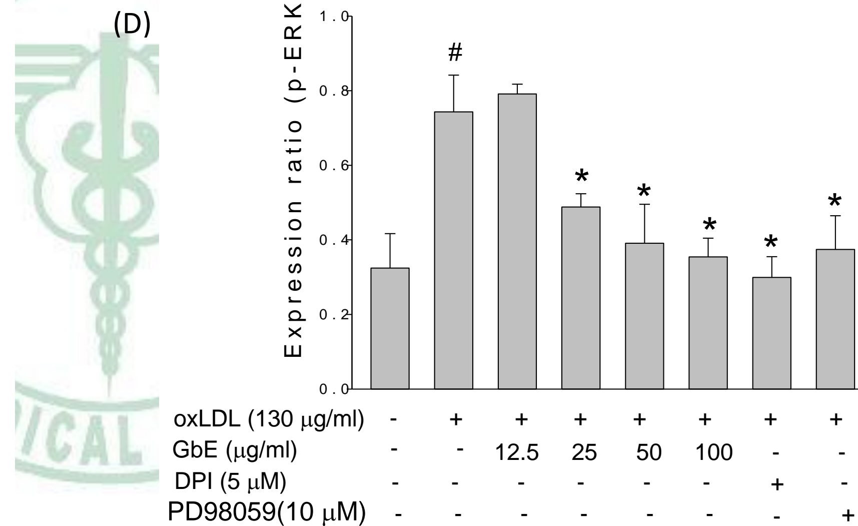
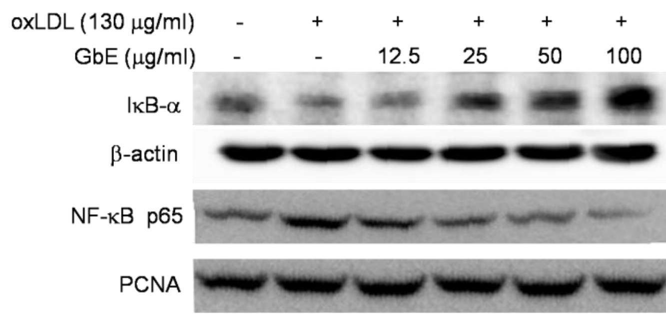
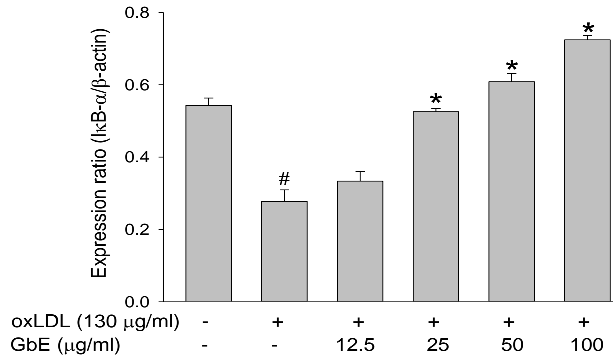


Fig.5 Effects of GbE on phosphorylation of p38MAPK and ERK





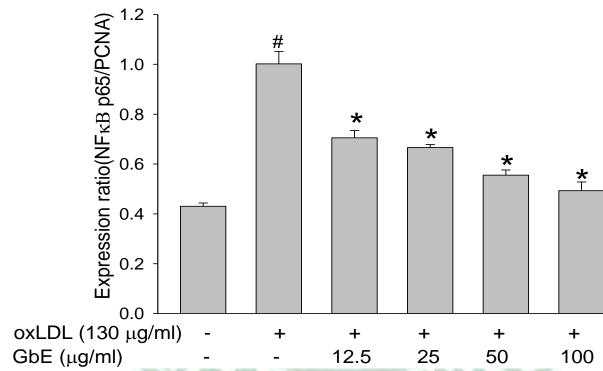
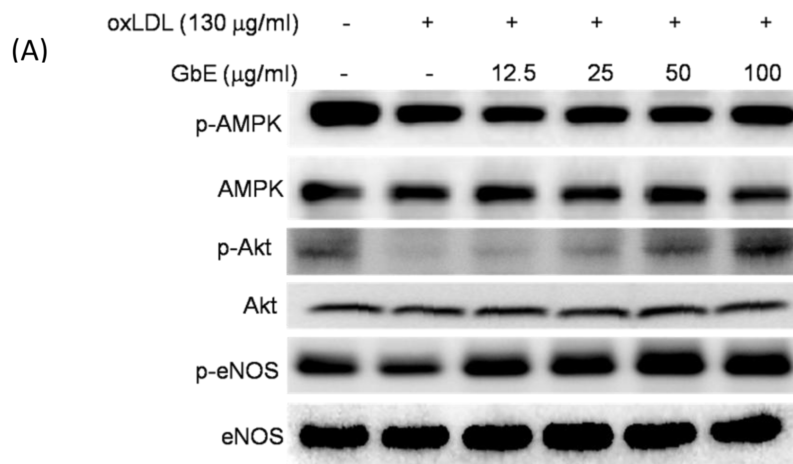
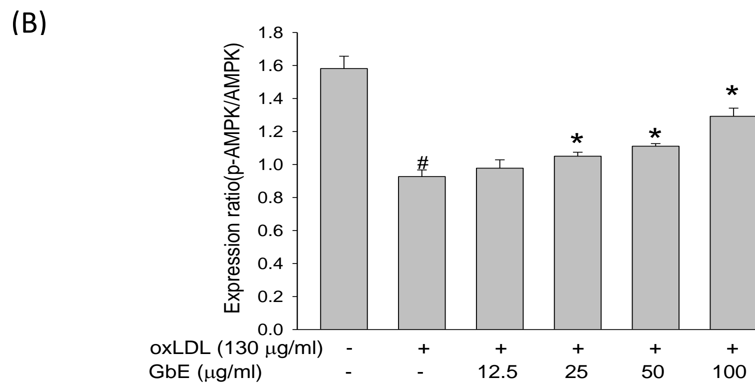
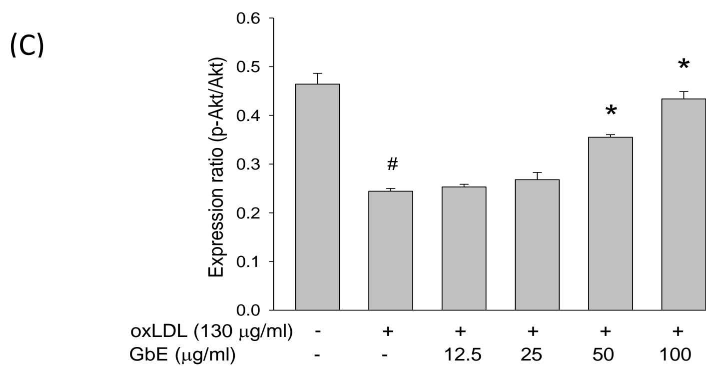


Fig.6 Effects of GbE on activation of NF-κB.







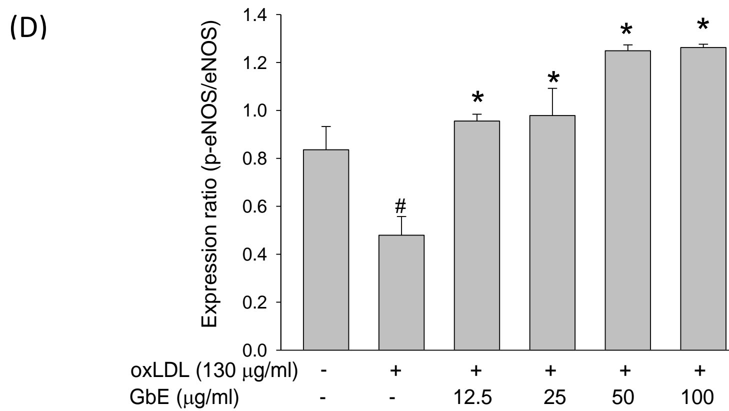
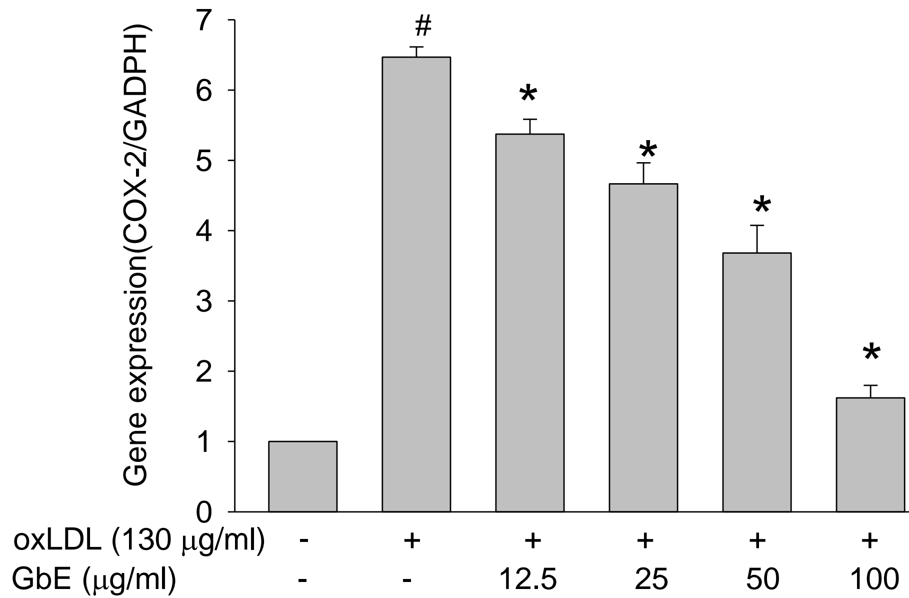
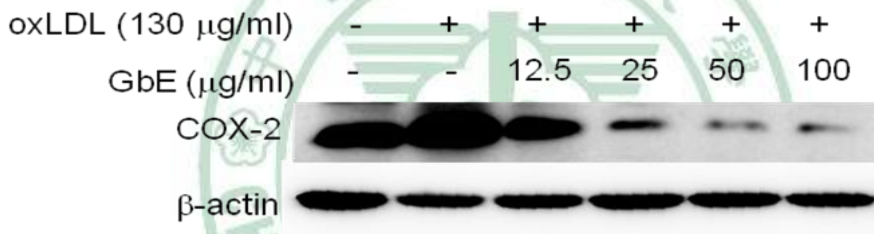


Fig.7 Effects of GbE on dephosphorylation of AMPK, Akt and eNOS

(A)



(B)



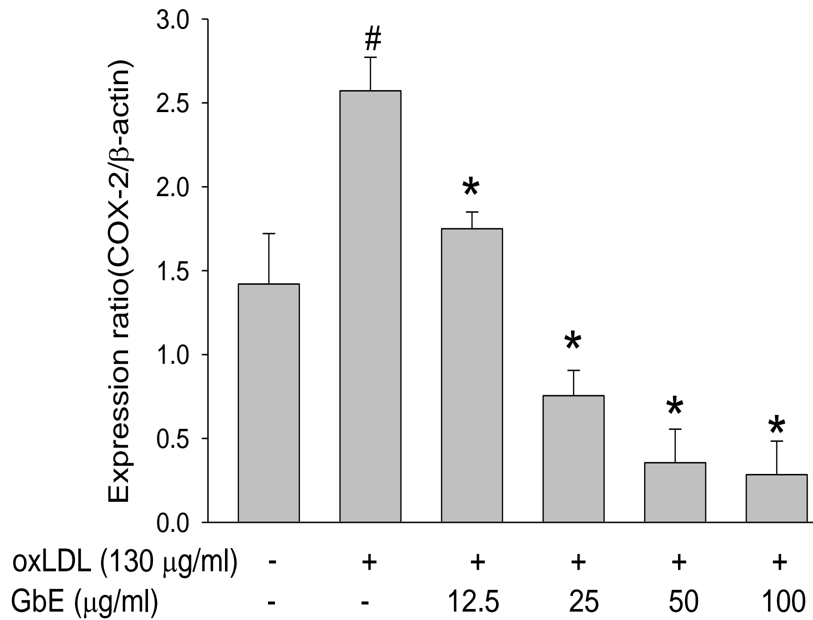


Fig.8 Effects of GbE on oxLDL-induced COX-2 expression through NF-κ B activation

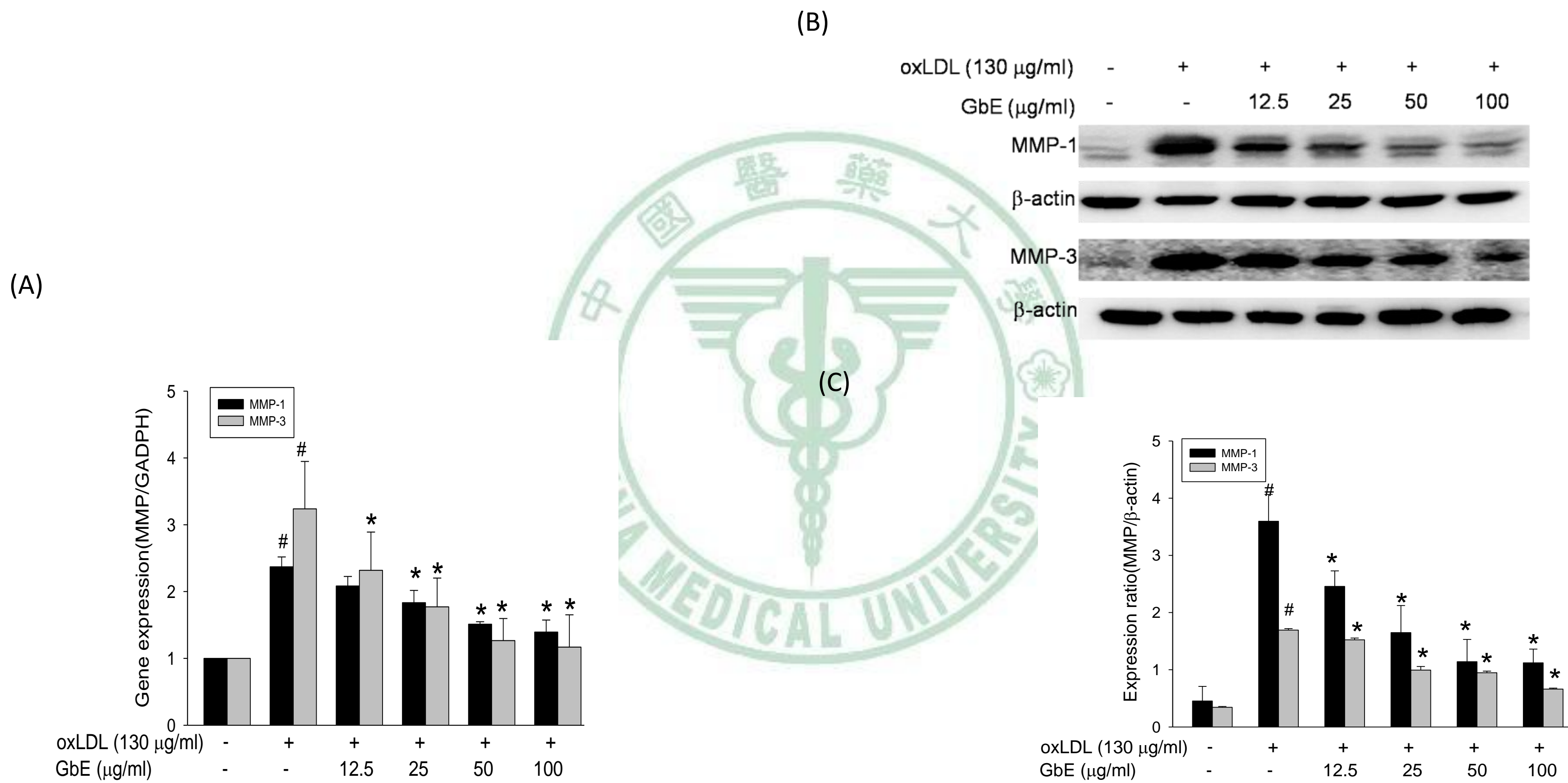
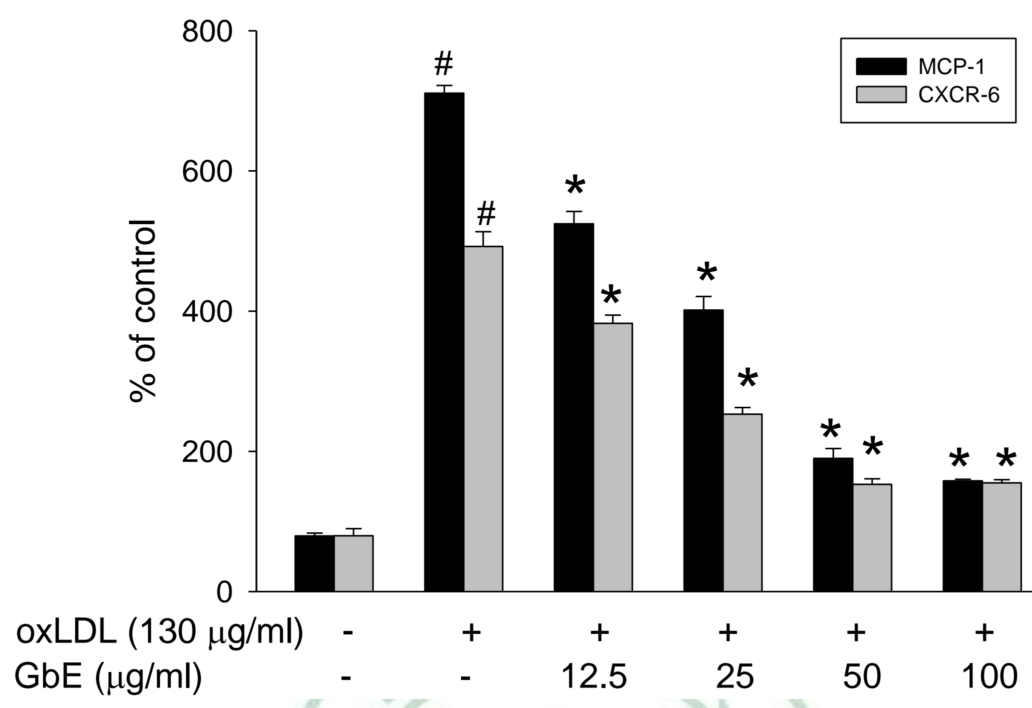


Fig.9 Effects of GbE on oxLDL-induced endothelial MMP-1 and MMP-3 gene and protein expression through NF-κ B activation



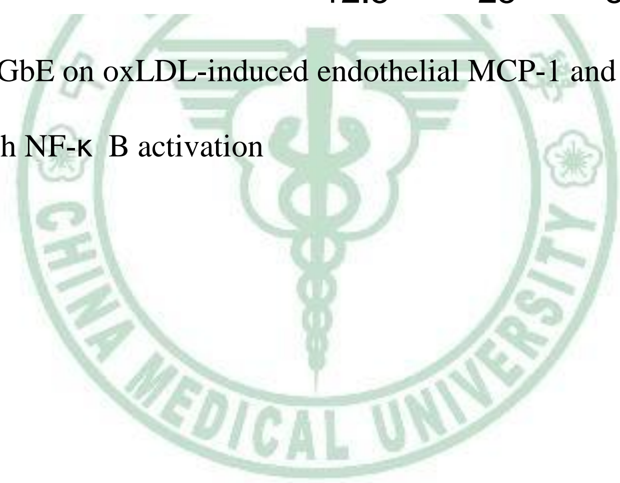


Fig.10 Effects of GbE on oxLDL-induced endothelial MCP-1 and CXCR-6 expression through NF-κ B activation

Legends

Fig.1. Inhibitory effects of GbE (Ginkgo biloba extract) on oxLDL-induced endothelial LOX-1(a lectin-like oxLDL receptor-1) gene (A) and protein (B) expression. The expression of LOX-1 mRNA and protein were analyzed by real-time PCR and Western blot respectively pretreated with GbE (12.5-100 μg/ml) or diphenyleneiodonium (DPI, 5 μM) for 2 hours followed by exposure to oxLDL(130 μg/ml) for a further 24 hours period in HUVECs. The levels of LOX-1 mRNA and protein were normalized to the level of GADPH and β-actin. Data of bar figure represent mean±SEM of 3 independent analyses. $^{\#}$ *P*<0.05 compared with control and **P*<0.05 compared with oxLDL-stimulated HUVECs.

Fig.2. Inhibitory effects of GbE on oxLDL-induced ROS generation in HUVECs. HUVECs were pretreated with GbE (12.5-100 μg/ml) for 2 hours, or anti-LOX-1 monoclonal antidoby (40 mg/ml), DPI (5 μM) followed by 1 hour incubation with fluorescent probe DCF-AM (10 μM), oxLDL(130 μg/ml) was then added to medium for 2 hours. (A) Fluorescence images show the ROS level in control cells (left) and HUVECs stimulated with oxLDL alone (middle) and in the presence of 200 μg/ml GbE (right). (B) Fluorescence intensity of cells was measured with a fluorescence microplate reader. Data of bar figure represent mean±SEM of 3 independent analyses. $^{\#}$ *P*<0.05 compared with control and **P*<0.05 compared with oxLDL-stimulated HUVECs.

Fig.3 Effects of GbE on oxLDL-induced p47phox and Rac-1 membrane translocation. The protein expression of p47phox and Rac-1 (A-C) were analyzed by Western blot pretreated with GbE (12.5-100 μg/ml) for 2 hours followed by oxLDL(130 μg/ml) for 30 min in HUVECs. Anti-flotillin-1 and anti-β-actin antibody were used for

normalization of membranes and cytosolic proteins, respectively. Data of bar figure represent mean±SEM of 3 independent analyses. # $P<0.05$ compared with control and *$P<0.05$ compared with oxLDL-stimulated HUVECs.

Fig.4. Effects of GbE on oxLDL-up-regulated iNOS, nitrotyrosine protein expression and oxLDL-enhanced Nitrite accumulation. The protein expression of iNOS and nitrotyrosine (A-C) were analyzed by Western blot pretreated with GbE (12.5-100 μg/ml) for 2 hours followed by oxLDL(130 μg/ml) for 24 hours in HUVECs. Anti-β-actin antibody was used for normalization of cytosolic proteins. Content of Nitrite was assayed using Gries reagent (D). Data of bar figure represent mean±SEM of 3 independent analyses. # $P<0.05$ compared with control and *$P<0.05$ compared with oxLDL-stimulated HUVECs.

Fig.5 Effects of GbE on phosphorylation of p38MAPK and ERK. The protein expression of phosphorylated and total p38MAPK and ERK (A,B) were analyzed by Western blot pretreated with GbE (12.5-100 μg/ml) for 2 hours prior to exposure to oxLDL(130 μg/ml) in HUVECs. Anti-p38 and anti-ERK antibody were used for normalization of cytosolic proteins. Data of bar figure represent means±SEM of 3 independent analyses. # $P<0.05$ compared with control and *$P<0.05$ compared with oxLDL-stimulated HUVECs

Fig.6 .Effects of GbE on activation of NF-κ B. The protein expression of Iκ B and NF-κ B(A-C) were analyzed by Western blot pretreated with GbE (12.5-100 μg/ml) for 30 min activated NF-κB in HUVECs. Anti-β-actin and anti-PCNA antibody were used for normalization of cytosolic and nuclear proteins, respectively. Data of bar figure represent means±SEM of 3 independent analyses. # $P<0.05$ compared with control and *$P<0.05$ compared with oxLDL-stimulated HUVECs

Fig.7. Effects of GbE on dephosphorylation of AMPK, Akt and eNOS. The protein

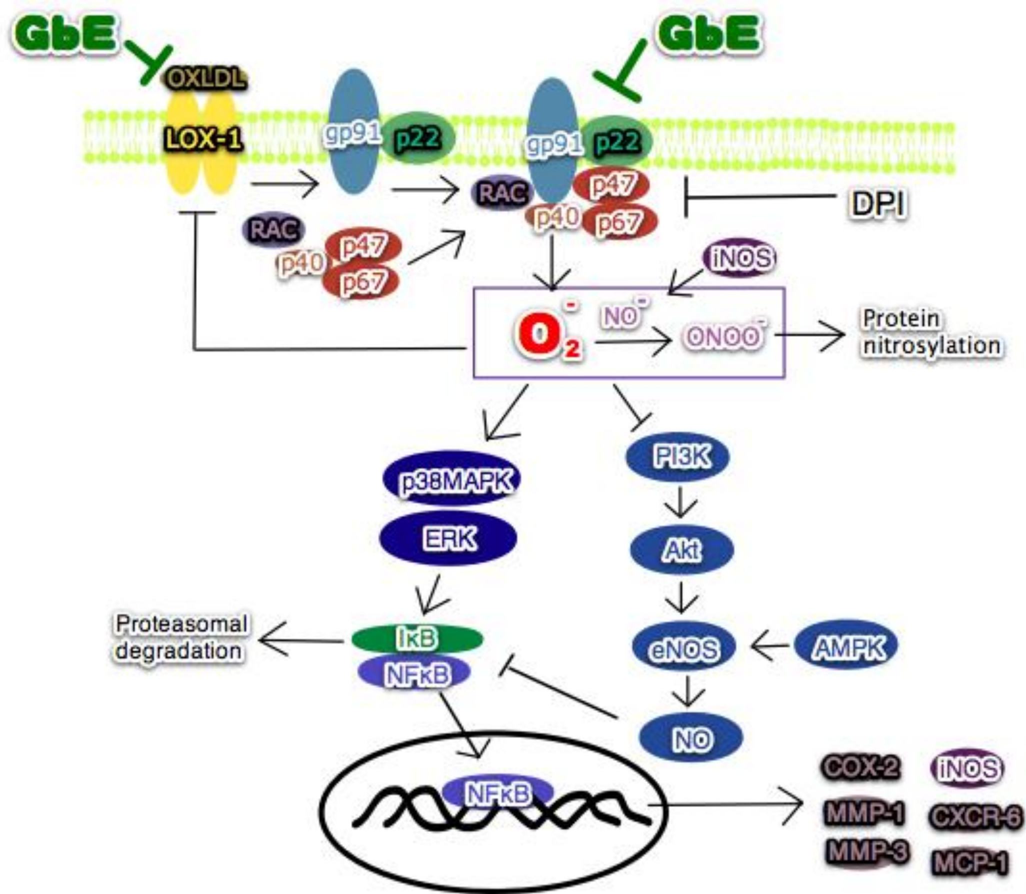
expression of dephosphorylated and total AMPK, Akt and eNOS (A-C) were analyzed by Western blot pretreated with GbE (12.5-100 μg/ml) for 2 hours prior to exposure to oxLDL(130 μg/ml) for 30 min in HUVECs. Data of bar figure represent means±SEM of 3 independent analyses. # *P*<0.05 compared with control and **P*<0.05 compared with oxLDL-stimulated HUVECs

Fig.8. Effects of GbE on oxLDL-induced COX-2 expression through NF-κB activation. The protein expression of COX-2 was analyzed by Western blot pretreated with GbE (12.5-100 μg/ml) for 2 hours prior to exposure to oxLDL(130 μg/ml) for 24 hours in HUVECs . Anti-β-actin antibody was used for normalization of cytosolic. Data of bar figure represent means±SEM of 3 independent analyses. # *P*<0.05 compared with control and **P*<0.05 vs. oxLDL treatment.

Fig.9 Effects of GbE on oxLDL-induced endothelial MMP-1 and MMP-3 gene and protein expression through NF-κB activation .The expression of MMP-1 and MMP-3 gene mRNA (A, B) and protein (C-E) were analyzed by real-time PCR and Western blot respectively pretreated with GbE (12.5-100 μg/ml) for 2 hours followed by exposure to oxLDL (130 μg/ml) for a further 24 hours period in HUVECs. At the end of the incubation period, cells were. The levels of MMP-1 and MMP-3 gene mRNA and protein were normalized to the level of GADPH and β-actin. Data of bar figure represent means±SEM of 3 independent analyses. # *P*<0.05 compared with control and **P*<0.05 compared with oxLDL-stimulated HUVECs

Fig.10 Effects of GbE on oxLDL-induced endothelial MCP-1 and CXCR-6 expression through NF-κB activation. HUVECs were incubated with oxLDL (130μg/ml) in the absence (control) or presence (oxLDL＋GbE) of 12.5-100 μg/ml GbE for 24 h. The histogram of cell surface expression of MCP-1 and CXCR-6 was

generated by flow cytometry. Data of bar figure represent means±SEM of 3 independent analyses. $^{\#}$ P<0.05 compared with control and *P<0.05 vs. oxLDL treatment.


GbE
OXLDL
LOX-1
gp91
p22
RAC
p47
p40
p67
DPI
iNOS
NO
ONOO
Protein nitrosylation
p38MAPK
ERK
PI3K
Akt
Proteasomal degradation
IκB
NFκB
eNOS
AMPK
NO
COX-2
MMP-1
MMP-3
CXCR-6
MCP-1

CHINA MEDICAL UNIVERSITY

References

1. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol.* 2005;25(1):29-38.
2. Hu C, Dandapat A, Sun L, Chen J, Marwali MR, Romeo F, Sawamura T, Mehta JL. LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet. *Cardiovasc Res.* 2008;79(2):287-293.
3. Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension.* 2004;44(3):248-252.
4. Li L, Renier G. The oral anti-diabetic agent, gliclazide, inhibits oxidized LDL-mediated LOX-1 expression, metalloproteinase-9 secretion and apoptosis in human aortic endothelial cells. *Atherosclerosis.* 2009;204(1):40-46.
5. Kaperonis EA, Liapis CD, Kakisis JD, Dimitroulis D, Papavassiliou VG. Inflammation and atherosclerosis. *Eur J Vasc Endovasc Surg.* 2006;31(4):386-393.
6. Widlansky ME, Gokce N, Keaney JF, Jr., Vita JA. The clinical implications of endothelial dysfunction. *J Am Coll Cardiol.* 2003;42(7):1149-1160.
7. Paravicini TM, Touyz RM. NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care.* 2008;31 Suppl 2:S170-180.
8. Touyz RM, Yao G, Schiffrin EL. c-Src induces phosphorylation and translocation of p47phox: role in superoxide generation by angiotensin II in human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 2003;23(6):981-987.

9. Takeya R, Sumimoto H. Regulation of novel superoxide-producing NAD(P)H oxidases. *Antioxid Redox Signal.* 2006;8(9-10):1523-1532.

10. Chen XP, Xun KL, Wu Q, Zhang TT, Shi JS, Du GH. Oxidized low density lipoprotein receptor-1 mediates oxidized low density lipoprotein-induced apoptosis in human umbilical vein endothelial cells: role of reactive oxygen species. *Vascul Pharmacol.* 2007;47(1):1-9.

11. Dunn S, Vohra RS, Murphy JE, Homer-Vanniasinkam S, Walker JH, Ponnambalam S. The lectin-like oxidized low-density-lipoprotein receptor: a pro-inflammatory factor in vascular disease. *Biochem J.* 2008;409(2):349-355.

12. Turpaev KT. Reactive oxygen species and regulation of gene expression. *Biochemistry (Mosc).* 2002;67(3):281-292.

13. Brigelius-Flohe R, Banning A, Kny M, Bol GF. Redox events in interleukin-1 signaling. *Arch Biochem Biophys.* 2004;423(1):66-73.

14. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene.* 1999;18(49):6853-6866.

15. Muller-Ladner U, Gay RE, Gay S. Role of nuclear factor kappaB in synovial inflammation. *Curr Rheumatol Rep.* 2002;4(3):201-207.

16. Chen M, Masaki T, Sawamura T. LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. *Pharmacol Ther.* 2002;95(1):89-100.

17. Shi X, Niimi S, Ohtani T, Machida S. Characterization of residues and sequences of the carbohydrate recognition domain required for cell surface localization and ligand binding of human lectin-like oxidized LDL receptor. *J Cell Sci.* 2001;114(Pt 7):1273-1282.

18. Kang BY, Hu C, Prayaga S, Khaidakov M, Sawamura T, Seung KB, Mehta JL. LOX-1 dependent overexpression of immunoglobulins in cardiomyocytes in response to angiotensin II. *Biochem Biophys Res Commun.* 2009;379(2):395-399.

19. Mehta JL, Chen J, Yu F, Li DY. Aspirin inhibits ox-LDL-mediated LOX-1 expression and metalloproteinase-1 in human coronary endothelial cells. *Cardiovasc Res.* 2004;64(2):243-249.

20. Chen XP, Zhang TT, Du GH. Lectin-like oxidized low-density lipoprotein receptor-1, a new promising target for the therapy of atherosclerosis? *Cardiovasc Drug Rev.* 2007;25(2):146-161.

21. Gaut JP, Heinecke JW. Mechanisms for oxidizing low-density lipoprotein. Insights from patterns of oxidation products in the artery wall and from mouse models of atherosclerosis. *Trends Cardiovasc Med.* 2001;11(3-4):103-112.

22. Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, Rigoni A, Pastorino AM, Lo Cascio V, Sawamura T. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. *J Biol Chem.* 2000;275(17):12633-12638.

23. Vergnani L, Hatrik S, Ricci F, Passaro A, Manzoli N, Zuliani G, Brovkovych V, Fellin R, Malinski T. Effect of native and oxidized low-density lipoprotein on endothelial nitric oxide and superoxide production : key role of L-arginine availability. *Circulation.* 2000;101(11):1261-1266.

24. Kunz J. Matrix metalloproteinases and atherogenesis in dependence of age. *Gerontology.* 2007;53(2):63-73.

25. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest.* 1994;94(6):2493-2503.

26. Kuzuya M, Kanda S, Sasaki T, Tamaya-Mori N, Cheng XW, Itoh T, Itohara S, Iguchi A. Deficiency of gelatinase a suppresses smooth muscle cell invasion and development of experimental intimal hyperplasia. *Circulation.* 2003;108(11):1375-1381.

27. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Libby P. Enhanced expression of vascular matrix metalloproteinases induced in vitro by cytokines and in regions of human atherosclerotic lesions. *Ann N Y Acad Sci.* 1995;748:501-507.

28. Kleijnen J, Knipschild P. Ginkgo biloba. *Lancet.* 1992;340(8828):1136-1139.

29. Hsiu-Chung Ou W-JL, I-Te Lee, Tsan-Hung Chiu, Kun-Ling Tsai, Chih-Ying Lin,and Wayne Huey-Herng Sheu. Ginkgo biloba extract attenuates oxLDL-induced oxidative functional damages in endothelial cells. *J Appl Physiol.* 2009;106:1674-1685.

30. Zhang Y CA, Li M, Chen C, Yao Q. Ginkgo biloba extract kaempferol inhibits cell proliferation and induces apoptosis in pancreatic cancer cells. *J Surg Res.* 2008;148: 17–23.

31. Diamond BJ SS, Feiwel N, Matheis RJ, Noskin O, Richards JA, Schoenberger NE. Ginkgo biloba extract: mechanisms and clinical indications. *Arch Phys Med Rehabil.* 2000;81: 668–678.

32. Lin SJ, Yang TH, Chen YH, Chen JW, Kwok CF, Shiao MS, Chen YL. Effects of Ginkgo biloba extract on the proliferation of vascular smooth muscle cells in vitro and on intimal thickening and interleukin-1beta expression after

balloon injury in cholesterol-fed rabbits in vivo. *J Cell Biochem.* 2002;85(3):572-582.

33. Tunali-Akbay T SG, Salvarli H, Sehirli O, Yarat A. Protective effects of Ginkgo biloba extract against mercury(II)-induced cardiovascular oxidative damage in rats. *Phytother Res.* 2007;21: 26–31.

34. Sastre J, Millan A, Garcia de la Asuncion J, Pla R, Juan G, Pallardo, O'Connor E, Martin JA, Droy-Lefaix MT, Vina J. A Ginkgo biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. *Free Radic Biol Med.* 1998;24(2):298-304.

35. Pincemail J, Dupuis M, Nasr C, Hans P, Haag-Berrurier M, Anton R, Deby C. Superoxide anion scavenging effect and superoxide dismutase activity of Ginkgo biloba extract. *Experientia.* 1989;45(8):708-712.

36. Maitra I, Marcocci L, Droy-Lefaix MT, Packer L. Peroxyl radical scavenging activity of Ginkgo biloba extract EGb 761. *Biochem Pharmacol.* 1995;49(11):1649-1655.

37. Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L. The nitric oxide-scavenging properties of Ginkgo biloba extract EGb 761. *Biochem Biophys Res Commun.* 1994;201(2):748-755.

38. Zhang L, Rui YC, Yang PY, Qiu Y, Li TJ, Liu HC. Inhibitory effects of Ginkgo biloba extract on vascular endothelial growth factor in rat aortic endothelial cells. *Acta Pharmacol Sin.* 2002;23(10):919-923.

39. Ou HC, Chou FP, Sheu WH, Hsu SL, Lee WJ. Protective effects of magnolol against oxidized LDL-induced apoptosis in endothelial cells. *Arch Toxicol.* 2007;81(6):421-432.

40. Napoli C, Quehenberger O, De Nigris F, Abete P, Glass CK, Palinski W.

Mildly oxidized low density lipoprotein activates multiple apoptotic signaling pathways in human coronary cells. *FASEB J.* 2000;14(13):1996-2007.

41. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-254.

42. Cross AR, Jones OT. The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase. *Biochem J.* 1986;237(1):111-116.

43. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J Immunol.* 1988;141(7):2407-2412.

44. Nagase M, Ando K, Nagase T, Kaname S, Sawamura T, Fujita T. Redox-sensitive regulation of lox-1 gene expression in vascular endothelium. *Biochem Biophys Res Commun.* 2001;281(3):720-725.

45. Dandapat A, Hu C, Sun L, Mehta JL. Small concentrations of oxLDL induce capillary tube formation from endothelial cells via LOX-1-dependent redox-sensitive pathway. *Arterioscler Thromb Vasc Biol.* 2007;27(11):2435-2442.

46. Becker LB. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovasc Res.* 2004;61(3):461-470.

47. Li D, Liu L, Chen H, Sawamura T, Ranganathan S, Mehta JL. LOX-1 mediates oxidized low-density lipoprotein-induced expression of matrix metalloproteinases in human coronary artery endothelial cells. *Circulation.* 2003;107(4):612-617.

48. Michell BJ, Griffiths JE, Mitchelhill KI, Rodriguez-Crespo I, Tiganis T, Bozinovski S, de Montellano PR, Kemp BE, Pearson RB. The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr Biol.* 1999;9(15):845-848.

49. Mehta JL, Sanada N, Hu CP, Chen J, Dandapat A, Sugawara F, Satoh H, Inoue K, Kawase Y, Jishage K, Suzuki H, Takeya M, Schnackenberg L, Beger R, Hermonat PL, Thomas M, Sawamura T. Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. *Circ Res.* 2007;100(11):1634-1642.

50. Marshall HE, Merchant K, Stamler JS. Nitrosation and oxidation in the regulation of gene expression. *FASEB J.* 2000;14(13):1889-1900.

51. Lan WJ, Zheng XX. Activity of Ginkgo biloba extract and quercetin on thrombomodulin expression and tissue-type plasminogen activator secretion by human umbilical vein endothelial cells. *Biomed Environ Sci.* 2006;19(4):249-253.

52. Wu YZ, Li SQ, Zu XG, Du J, Wang FF. Ginkgo biloba extract improves coronary artery circulation in patients with coronary artery disease: contribution of plasma nitric oxide and endothelin-1. *Phytother Res.* 2008;22(6):734-739.