·Basic Research·

Effect of EGb761 on light-damaged retinal pigment epithelial cells

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Abstract

• AIM: To investigate the protective mechanism of Gingko Biloba extract (EGb761) on the ability of retinal pigment epithelial (RPE) cells to resist light-induced damage in a comparative proteomics study.

• METHODS: Human RPE cells (ARPE-19) were randomly distributed to one of three groups: normal control (NC group) and light-damaged model without or with EGb761 group (M and ME groups, respectively). The light-damaged model was formed by exposing to white light (2 200±300)lx for 6h. The RPE cells in ME group were conducted with EGb761 (100μg/mL) before light exposure. The soluble cellular proteins extracting from each groups were separated by two-dimensional electrophoresis and stained by silver staining. Different proteins in the profiles of the gels were analyzed by Image Master Software. Two-fold expressing protein spots were identified by Matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry.

• RESULTS: NC, M and ME groups displayed 1 892±71, 2 145±23 and 2 216±85 protein spots, respectively. We identified 33 proteins with different expression levels between the NC and M groups, 25 proteins between the M and ME groups, and 11 proteins between the NC and ME groups. MALDI-TOF/TOF mass spectrometry successfully identified 16 proteins, including metabolic enzymes, cytoskeletal proteins, anti-oxidation proteins, and others.

• CONCLUSION: Differences in some important proteins, such as cathepsin B, heat shock protein, and cytochrome C reductase, indicated that multiple pathways may be induced in light-damaged RPE cells and the protective effect of EGb761.

• **KEYWORDS:** EGb761; retinal pigment epithelial cells; light damage; protein; mass spectrographic analysis
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INTRODUCTION

Retinal pigment epithelial (RPE) cells are highly differentiated, polarized, epithelial monolayer cells. They participate in visual pigment recycling, serve as a major component of the blood-retinal barrier, and synthesize numerous enzymes to digest debris abandoned by photoreceptors [1-3]. Insufficiency of RPE-specific functions and dedifferentiation will cause a series of ocular lesions, including proliferative vitreoretinopathy and secondary or degenerative retinal disease, such as age-related macular degeneration and Best's disease[4].

Excessive light exposure may worsen age-related macular degeneration or other retinal degeneration disorders [5]. Moreover, it has been reported that exposure to strong light during ophthalmologic examination or surgery can result in photic maculopathy[6]. Because the vertebrate retina serves as a light processor, free radicals can be produced as part of normal processes [7]. However, because the retina contains a high level of polyunsaturated fatty acids, which are selective substrates for peroxidation, it is vulnerable to attacks by free radicals [8]. By now, it is clear that light-induced retina damage is influenced by light intensity, duration, and wavelength; the distribution of absorbing chromophores; and levels of oxygen radical scavengers, such as ascorbic acid[9-14]. However, the specific proteins involved remain unknown.

Ginkgo biloba is a Chinese herbal medicine extracted from the leaves of the maidenhair tree, and it is widely used in diverse medical treatments. It has been reported to increase ocular blood flow velocity in the ophthalmic artery of glaucoma patients [15]. Juárez *et al* [16] found that it is an effective angiostatic inhibitor in a study of premature retinopathy. Ranchon *et al* [17] reported that intraperitoneal injection of ginkgo biloba extract (EGb761) could preserve retinal function after light-induced retinal damage in rats, but

the mechanism is not known. EGb761 contains flavonoid substances, such as the ginkgo flavone glycosides, which have been verified to prevent the cellular generation of free radicals and serve as free radical scavengers[18].

To investigate the potential protective effect of EGb761 against light-induced RPE cell degeneration, we studied the expression levels of different proteins in a model of light-damaged human RPE cells using a proteomic approach based on two-dimensional (2-D) gel electrophoresis and mass spectrometry.

MATERIALS AND METHODS

Cell Culture The human RPE cell line ARPE-19 was purchased from the American Type Culture Collection (ATCC, Rockville, MA, USA). ARPE-19 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagles medium and Ham's F-12 medium (DMEM/F-12; Gibco), supplemented with 10% fetal bovine serum (Gibco), 100U/mL penicillin, and 100μg/mL streptomycin. Cells were maintained at 37℃ with a humidified atmosphere of 95% air and 5% CO_2.

Cell Intervention When cell culture flasks were filled with 90% ARPE-19, cells were separated into normal control group (NC group), light-induced damage model group (M group) and model with EGb761 (ME group) at random. Cells were exposed to cold white (2 200±300)lx light 6h to create light damage model. RPE in ME group was treated with EGb761 (100μg/mL) before light exposure. Cells of the three groups were cultured for 24h before soluble protein extraction.

Sample Preparation ARPE-19 cells were washed three times with phosphate-buffered saline (PBS). Subsequently, cells were lysed in lysis buffer consisting of 7mol/L urea, 2mol/L thiourea, 4% 3-3-cholamidopropyl dimethylammonio-l-propanesulfonate (CHAPS; Dojindo, Kumamoto, Japan) and 1% dithiothreitol (DTT), and collected using a cell scraper. Lysates were stored at -80℃ until use. The protein concentrations were measured by Bradford assay using BSA as a standard solution.

2 -D Polyacrylamide Gel Electrophoresis Lysate containing 100μg total protein was used for each gel. Immobilized pH gradient (IPG) Strips (pH3-10, 13cm) (Amersham Biosciences, Sweden) were rehydrated overnight with in rehydration buffer containing the protein samples. Isoelectric focusing (IEF) was performed in three steps: 500V for 1h, 1 000V for 1h and 8 000 V for 5h. The gel strips were equilibrated for 15min in equilibration buffer (50mmol/L Tris-HCl, pH8.8, 6mol/L urea, 2% sodium dodecyl sulfate (SDS), 30% glycerol, and 1% DTT). This step was repeated using the same buffer with 4% iodoacetamide in place of 1% DTT. The strips were then subjected to the second-dimensional electrophoresis after transfer onto 12.5% SDS-polyacrylamide gels (PAGE). Electrophoresis was performed using the Hofer SE 600 system (Amersham) at 30mA per gel for 40min, followed by 60mA until the bromophenol blue reached the end of the gel. At least three replicates were performed for each sample.

Gel Staining and Image Analysis Of nine silver-stained gels from each sample, the three best focusing results were selected for imaging analysis using a Bio-Rad GS710 scanner, and image master software (Amersham).

In-gel Tryptic Digestion Protein spots were excised from preparative gels and destained with 100mmol/L NH_4HCO_3 and 30% acetonitrile (ACN). After removing the destain buffer, the gel pieces were lyophilized and rehydrated in 30μL of 50mmol/L NH_4HCO_3 containing 50ng of trypsin (sequencing grade; promega, WI). After digestion overnight at 37℃, the peptides were extracted three times with 0.1% trifluoroacetic acid (TFA) in 60% ACN. Extracts were pooled together and lyophilized. The resulting lyophilized tryptic peptides were dissolved in 5mg/mL a-cyano-4-hydroxycinnamic acid (CHCA) containing 0.1% TFA and 50% ACN. A protein- free gel piece was treated the same as above and used as a control to identify autoproteolytic products derived from trypsin.

Matrix-assisted Laser Desorption Ionization-time of Flight-time of Flight (MALDI-TOF/TOF) Mass Spectrometry Analysis and Database Searching Mass spectra were acquired on a MALDI-TOF/TOF tandem mass spectrometer, the Bruker-Daltonics AutoFlex TOF-TOF LIFT. The instrument was operated in the delayed extraction and positive-ion linear mode with the following parameters: 20kV acceleration voltage, 95% grid voltage, 100ns delay time, and 500m/z low-mass gate. For acquisition of a mass spectrometric peptide map, a 1-μL aliquot from the peptide extracts was premixed with 1μL of matrix (10mg/mL CHCA in 35% ACN and 0.1% TFA) and spotted onto a MALDI target plate. Measurements were externally calibrated with a standard peptide mixture of angiotensin Ⅱ ($[M+H]^+$1046.54) and angiotensin Ⅰ ($[M+H]^+$1296.68), and internally recalibrated with peptide fragments arising from autoproteolysis of trypsin. Both MS and MS/MS data were acquired with a N_2 laser at a 25-Hz sampling rate. The monoisotopic masses were processed for identification. For MS/MS spectra, the peaks were calibrated by default and smoothed. All peaks were deisotoped.

MS/MS was performed using the MASCOT program. The data were searched based on protein databases in NCBI (updated in February 16, 2006), which contained 2 464 940 sequences. The search was performed by using other Metazoa as taxonomy, which contained 154 412 sequences. The other search parameters were enzyme of specificity strict trypsin; one missed cleavage; fixed modifications of carbamidomethyl (C); oxidation (Met); peptide tolerance of 100×10^{-6}; fragment mass tolerance of ±0.5Da; peptide charge of 1+; and monoisotopic. Only significant hits, defined by

MASCOT probability analysis (P<0.05), were accepted.

Peptide mass fingerprinting (PMF) was performed using the search engine of MS-Fit in the Protein Prospector v 4.0.6 package (http://prospector.ucsf.edu/, Protein Prospector, San Francisco, CA, USA). The Swiss-Prot.2007.01.21 and NCBInr.2006.02.16 database were used for protein identification.

RESULTS

Using Image Master software, averages of 1 892±71, 2 145± 23, and 2 216 ±85 spots were detected on gels from the normal control (NC), light-damaged model without EGb761 (M), and light-damaged model with EGb761 (ME) groups, respectively. The three gel images from each sample were analyzed to determine quantitative and qualitative differences in protein expression. After the experiments were repeated in triplicate, 33 reproducible differences were found between the M and NC groups, of which 23 proteins were up-regulated and the other 10 proteins were down-regulated (Figure 1). Twenty-five reproducible differences were observed between the ME and M groups, of which 3 proteins were up-regulated and the other 22 proteins were down-regulated (Figure 2). Eleven reproducible differences were observed between the ME and NC groups; four and seven proteins were up- and down-regulated, respectively (Figure 3). Among these, sixteen spots could be identified by matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF), including metabolic enzymes, cytoskeletal proteins, anti-oxidation protein, and others (Tables 1, 2).

Cathepsin B and heat shock protein (HSP) 70 expression were up-regulated in the M group and down-regulated in the ME group. Conversely, cytochrome C reductase was down-regulated in the M group and up-regulated in the ME group.

DISCUSSION

ARPE-19 is a human RPE cell line that can be spontaneously generated during initial RPE culturing. Although the exact mechanisms underlying ARPE-19 immortality are still unclear, it is a valuable source of human RPE cells. These cells display a highly epithelial morphology with a hexagonal cobblestone layer.

RPEs are essential for vision because of their roles in maintaining photoreceptors. Previous experimental studies on light-induced damage in rodent eyes have clarified that light exposure causes photoreceptor damage and initiates the apoptotic pathway [19-21]. Intense light exposure causes lipid peroxidation of retinal tissues, and oxidative stress is likely to be involved in the pathogenesis of light-induced retinal damage [9,22,23]. To date, several possible but controversial pathways, such as increased production of reactive oxygen species and photosensitization of retinal chromophores, have been proposed to be the mechanisms of light-induced lipid peroxidation [24,25]. EGb761 has been reported to increase ocular blood flow and work as an effective angiostatic inhibitor [26]. However, it is difficult to assess the pharmacokinetic effects of EGb761 because it contains many active ingredients. Indeed, interactions among them are likely and may induce different mechanisms.

Our proteomic analysis demonstrated that cathepsin B, a cysteine protease, was up-regulated in the M group and down-regulated in the ME group. Cathepsins are cysteine

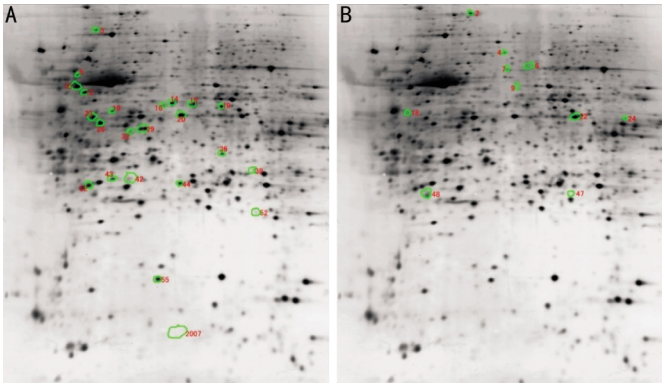

Figure 1 Differential expressing proteins on 2 –DE gels between the light –induced damage model group and the normal control group stain by Silver A: Twenty-three proteins were up-regulated; B: Ten proteins were down-regulated.

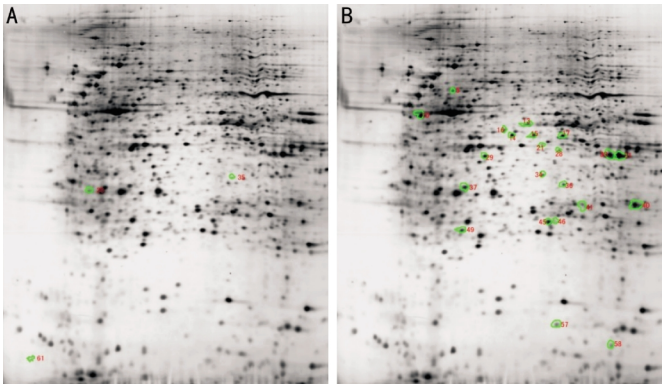

Figure 2 Differential expressing proteins on 2 –DE gels between the model with EGb761 group and the light–induced damage model group stain by Silver A: Three proteins were up-regulated; B: Twenty-two proteins were down-regulated.

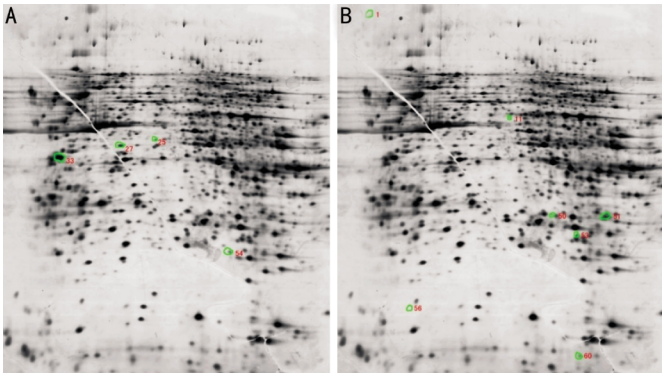

Figure 3 Differential expressing proteins on 2 –DE gels between the model with EGb761 group and the normal control group stain by Silver A: Four proteins were up-regulated; B: Seven proteins were down-regulated.

Table 1 Description of identified proteins

Spot No.	Mascot protein name	NCBI GI No.	Mascot MW(Da)/pI	Mowse score	Sequence coverage (%)	No. of peptides
10	Heat shock 70kDa protein 8	5729877	71086/5.37	196	4	3
12	Heat shock protein	386785	70115/5.42	144	5	3
14	Carbonate dehydratase	693933	47427/7.01	106	10	8
17	Pyruvate kinase	35505	58421/7.58	185	11	4
22	Cathepsin B complex with dipeptidyl nitrile inhibitor	24158605	29584/5.34	74	27	7
24	Annexin A2 isoform 2	4757756	38812/7.57	80	5	2
26	Hsp89-alpha-delta-N	3287498	85027/4.94	116	3	2
29	Valosin-containing protein	6005924	89962/5.14	119	3	2
34	Elongation factor Tu	704416	49857/7.70	265	9	4
36	GRP78 precursor	386758	72187/5.03	184	9	4
40	Heat shock 70kDa protein 8 isoform 1	5729877	71086/5.37	328	11	5
45	Triosephosphate isomerase	136066	26899/7.01	138	11	2
47	Cytochrome C reductase	45768728	29963/8.55	38	5	1
48	Lysosomal proteinase cathepsin B	181178	23643/5.19	114	15	2
55	Stathmin 1	5031851	17292/5.76	122	24	3
62	Annexin I	4502101	38922/6.57	340	18	3

Table 2 Differential protein expression between the normal control group (NC group), light-induced damage model group (M group) and model+EGb761 group (ME group)

Spot No.	Mascot protein name	Expression patterns		
		M-N	E-M	E-N
10	Heat shock 70kDa protein 8	↑	↓	↓
12	Heat shock protein	↑	↓	↓
14	Carbonate dehydratase	↑	↓	↑
17	Pyruvate kinase	↑	↓	↑
22	Cathepsin B complex with dipeptidyl nitrile inhibitor	↑	↓	↓
24	Annexin A2 isoform 2	↓	↓	↓
26	Hsp89-alpha-delta-N	↑	↓	↓
29	Valosin-containing protein	↑	↓	↓
34	Elongation factor Tu	↑	↓	↑
36	GRP78 precursor	↑	↓	↑
40	Heat shock 70kDa protein 8 isoform 1	↑	↓	↓
45	Triosephosphate isomerase	↑	↓	↑
47	Cytochrome C reductase	↓	↑	↑
48	Lysosomal proteinase cathepsin B	↑	↓	↓
55	Stathmin 1	↑	↓	↓
62	Annexin I	↓	↓	↓

M-N: The light-induced damage model group *vs* the normal control group; E-M: Model+EGb761 group *vs* the light-induced damage model group; E-N: Model+EGb761 group *vs* the normal control group.

endopeptidases that belong to the family of papain-like proteolytic enzymes principally located in the endosomal/lysosomal compartment [27]. When tumors arise, increased cathepsin B expression is associated with angiogenesis, and it is speculated to promote extracellular matrix remodeling to permit neovascularization [27-30]. We theorise that light induced oxidative reactions that can damage the lysosome membrane, while cathepsins contribute to the degradation of collagens, connective tissue proteins, and certain native enzymes. Thus, over-expression of cathepsin B protein may be associated with inflammation, necrosis, and apoptosis.

Our results demonstrate that cytochrome C reductase was down-regulated in the M group and up-regulated in the ME group. Cytochrome C oxidase subunit III (CO-III) is the terminal enzyme of the mitochondrial respiratory chain and catalyzes the transfer of electrons from reduced cytochrome C to molecular oxygen and is therefore a key enzyme for ATP production. In addition, histochemistry and immunohistochemistry have revealed that CO-III is found in all retinal layers[31]. The results of the present study show that cytochrome C reductase content was decreased in the M

group and increased in the ME group. These results indicate that decreased cytochrome c reductase expression has a negative effect on electron transport, oxidative phosphorylation, and ATP/energy production in the retina.

We also found an increase in HSP70 in the M group. Although it is constitutively expressed in cells, HSP70 is increased in response to various types of stress, especially oxidant-induced injury. Previous studies have suggested that multiple expressions of HSP70 are involved in responding to light-induced oxidative stimuli[32-34].

It can be assumed that the anti-oxidant action of EGb761 is due to its flavonoid glycosides, which can scavenge oxygen free radicals and lipid peroxides [35,36]. Thus, EGb761 can protect against light-induced retinal damage. In addition, ginkgo flavone glycosides, the major content of the extract, contains other substances of some interest, such as organic acids, that can enhance water solubility. Thus, unlike ascorbic acid, glutathione, and uric acid, EGb761 has both lipophilic and hydrophilic characteristics. Maitra *et al*[37] demonstrated that EGb761 scavenges peroxyl radicals generated in lipid and aqueous environments. Furthermore, EGb761 has also been found to exert a membrane-stabilizing action[38].

In summary, our results suggest that multiple pathways may be involved in the light-induced damaged of RPE cells and indicate a protective effect of EGb761. Specifically cathepsin B, HSP70, and cytochrome C reductase appear to play roles. However, it should be noted that the interpretation and clinical relevance of this study are limited given the potential differences between cultured RPE cells and *in vivo* pseudometaplastic RPE cells; the latter are exposed to complex interactions with the intraocular environment. Further research on the response of RPE cells to light-induced damage and EGb761 treatment *in vivo* using this technique may reveal a specific protective mechanism against light-induced damage.

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