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Up-Regulation of Crystallins is Involved in the Neuroprotective Effect of Wolfberry on Survival of Retinal Ganglion Cells in Rat Ocular Hypertension Model

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ABSTRACT

Wolfberry (fruit of *Lycium barbarum* Linn) has been known for balancing 'Yin' and 'Yang' in the body, nourishing the liver and kidney, improving visual acuity for more than 2,500 years in oriental countries. The active components in wolfberry include *L. barbarum* polysaccharide (LBP), zeaxanthine, betaine, cerebroside and trace amounts of zinc, iron, and copper. Each of them confers distinct beneficial effects and together they help to explain widespread use of wolfberry in the eastern world. Earlier study reported the neuroprotective effects of LBP on retinal ganglion cell (RGC) in an experimental model of glaucoma and the underlying in vivo cellular mechanisms of LBP neuroprotection deserve further exploration. In this study, we adopted proteomics, functional genomics, to evaluate pharmacological effects of LBP on the neuronal survival pathways. Among the significantly changed proteins induced by LBP feeding on ocular hypertension (OH) retinas, only proteins in crystallin family were focused in this study. The proteomic results were further confirmed using the Western blotting of the retinas and immunohistochemical staining of the retinal sections. We demonstrated that neuroprotective effect of—wolfberry extract—LBP on the survival of RGCs may be mediated via direct up-regulation of neuronal survival signal βB2-crystallin. J. Cell. Biochem. 110: 311–320, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: WOLFBERRY; GLAUCOMA; CRYSTALLIN; NEUROPROTECTION; RETINA; RETINAL GANGLION CELLS

Wolfberry (fruit of *Lycium barbarum* Linn, in the family Solanaceae) is known as Fructus Lycii and Wolfberry in the West, and Gouqizi or Kei Tze in Asia. The fresh fruits are small red berries which are sweet in taste. It has been regarded as an upper Chinese medicine, indicating to be one of the ingredients in Chinese cuisine or formulated Chinese medicine. In traditional Chinese medicine literature, it has been known for balancing 'Yin' and 'Yang' in the body, nourishing the liver and kidney, improving visual acuity for more than 2,500 years. It is used to treat poor vision, dizziness, tinnitus, soreness of limbs and knees, nocturnal emission [Xie, 1956]. With the help of modern chemical analytic methods, we know that the active components in wolfberry include *L. barbarum* polysaccharide (LBP), zeaxanthine, betaine, cerebroside and trace amounts of zinc, iron, and copper [Chang and But, 1983; Chai et al., 1986; Kim et al., 1999]. Each of them confers distinct beneficial effects and together they help to explain widespread use of wolfberry in the eastern world [Chang and So, 2008; Ho et al., 2010b]. For example, zeaxanthin and lutein can improve visual

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acuity [Lam and But, 1999]; reduce the risk of age-related macular degeneration [Gale et al., 2003; Moeller et al., 2006] and directly protect against photoreceptor apoptosis induced by oxidative stress [Thomson et al., 2002; Chucair et al., 2007]. Since the major content of wolfberry is LBP (about 40%), research in wolfberry often focuses on these water soluble fractions. It has been shown that intraperitoneal injection of LBP (60 g/0.5 ml) can reduce stroke index and neurological score in a mouse cerebral ischemia and reperfusion model [Song et al., 1995]. LBP can improve cognitive functions by enhancing the spontaneous electrical activity of the hippocampus [Peng et al., 2002]. Our earlier study reported the neuroprotective effects of LBP on retinal ganglion cell (RGC) in an experimental model of glaucoma [Chan et al., 2007]. However, the underlying in vivo cellular mechanisms of LBP neuroprotection deserve further exploration.

Crystallins are the dominant structural proteins in the lens and augment the refractive power of the transparent lens tissue. Crystallin gene families are divided into major ones: α-crystallins, and βγ-crystallins [Andley, 2007]. Alpha B crystallin was the first one to be found in non-lens tissues [Bhat and Nagineni, 1989; Dubin et al., 1989] and later proved to be functional chaperones that protect other proteins against thermal insults [Horwitz, 1992; Merck et al., 1993]. Members of the βγ-crystallins have also been detected outside of lens [Head et al., 1991, 1995; Jones et al., 1999; Magabo et al., 2000]. Increased expressions of crystallins have been suggested as a cellular response mechanism against stress [Kamradt et al., 2001, 2002, 2005; Whiston et al., 2008; Yaung et al., 2008]. A primary objective in this study is using proteomics, functional genomics, to evaluate pharmacological effects of LBP on the neuronal survival of RGCs in a chronic ocular hypertension (OH) model. Proteomic elucidated the global analysis of gene expression and their functions at the protein level and the proteomic results were further confirmed using the Western-blot analysis of the retinas and immunohistochemical staining of the retinal sections. We demonstrated that neuroprotective effect of LBP on the survival of RGCs under OH may involve direct up-regulation of neuronal expression of crystallins, especially βB2-crystallin.

MATERIALS AND METHODS

PREPARATION AND CHEMICAL ANALYSIS OF LBP

The wolfberry originated from NingXia Huizu Autonomous Region, the People's Republic of China. The simplified extraction scheme from wolfberry has been reported by our group [Yu et al., 2007]. Two grams of LBP can be extracted from 10 kg of wolfberry. The LBP was found to posses 18% (w/w) neutral sugars and 8.6% (w/w) protein. The carbohydrate in LBP contains (value expressed in mol%) mainly glucose (47.7%), arabinose (16.1%), galacturonic acid (14.4%), and galactose (12.9%). The amino acid component of LBP (value expressed in mol%) consists of aspartic acid (27.38%), glutamic acid (13.02%), and cysteine (10.49%) as major amino acids.

ANIMAL GROUPING

Sixty adult female Sprague–Dawley (SD) rats (250–280 g) were obtained from the Laboratory Animal Unit of the LKS Faculty of medicine in the University of Hong Kong, and were maintained in a temperature-controlled room with a 12 h light/dark cycle throughout the observation period. Prior to measuring intraocular pressure (IOP) or any other operation, the rats were anesthetized with an intra-peritoneal injection of a ketamine/xylazine mixture (ketamine 80 mg/kg and xylazine 8 mg/kg) (Alfasan, Woerden, Holland). All operations were performed under an operating microscope (Olympus OME, Tokyo, Japan). The animals were handled according to the protocol for the use of animal in research approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong and the Association for Research in Vision and Ophthalmology, USA, statements for the use of animals in Ophthalmic and Vision Research.

Experimental groups in the LBP study are shown in Table I. The effective LBP dose used in this study was 1 mg/kg, chosen from the dose response study done by our previous study [Chan et al., 2007]. The LBP powder was dissolved in 0.01 M sterilized PBS (pH 7.4). Animals were fed daily through a nasogastric tube with 1 ml of either PBS or 1 mg/kg LBP, starting from 1 week prior to the first laser photocoagulation until euthanization (Fig. 1).

OCULAR HYPERTENSION MODEL

Ocular hypertension (OH) was induced in the right eye of each animal using laser photocoagulation according to our previous publications [Ji et al., 2004; Chan et al., 2007; Chiu et al., 2007]. Briefly, the limbal vein and the three radial episcleral aqueous humor drainage veins (superior nasal, superior temporal and inferior temporal) were photocoagulated using an Argon laser (Ultima 2000SE Argon Laser, Coherent, USA). About 60 laser spots (power, 1,000 mV; spot size, 50–100 μm; duration, 0.1 s) around the limbal vein (except the nasal area) and 15–20 laser spots on each episcleral aqueous humor drainage vein were applied. To maintain high IOP, a second laser treatment at the same settings was applied 7 days later

TABLE I. Experimental Groups in LBP Study

Treatment	Animal number	Survival time after the first laser photocoagulation	Objectives
Normal rats	12	Inapplicable	Normal control
PBS fed normal rats	6	Inapplicable	PBS control
LBP fed normal rats	6	Inapplicable	LBP control
PBS fed with right eye OH	6	2 days	Proteomic and Western blotting
PBS fed with right eye OH	12	14 days	Proteomic, RGC survival and IHC
LBP fed with right eye OH	6	2 days	Proteomic and Western blotting
LBP fed with right eye OH	12	14 days	Proteomic, RGC survival, and IHC

LBP, *Lycium barbarum* polysaccharide; OH, ocular hypertension; RGC, retinal ganglion cell; IHC, immunohistochemistry.

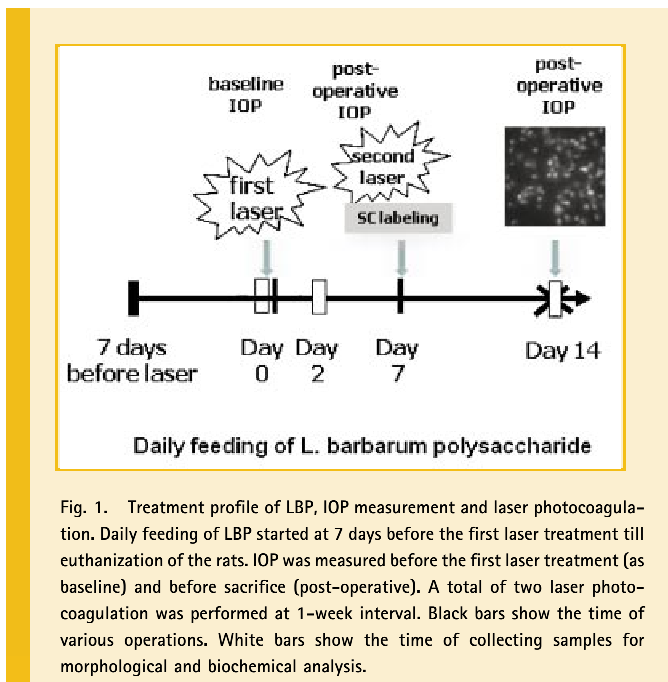

Fig. 1. Treatment profile of LBP, IOP measurement and laser photocoagulation. Daily feeding of LBP started at 7 days before the first laser treatment till euthanization of the rats. IOP was measured before the first laser treatment (as baseline) and before sacrifice (post-operative). A total of two laser photocoagulation was performed at 1-week interval. Black bars show the time of various operations. White bars show the time of collecting samples for morphological and biochemical analysis.

in the 14 days groups. After each laser treatment, ophthalmic Tobrex ointment (3% tobramyxin, Alcon-Couvreur, Belgium) was applied topically to prevent infection.

MEASUREMENT OF IOP

IOP was measured with a Tonopen XL tonometer (Mentor®, Norwell, USA) before the first laser treatment and every subsequent week until the rats were sacrificed. To avoid diurnal variation and effect of anesthesia, all IOP measurements were taken at 10 o'clock in the morning and within 15–30 min after anesthesia using ketamine and xylazine mixture (i.p.). Before each measurement, one drop of proparacaine hydrochloride (0.5% alcaine, Alcon-Couvreur, Belgium) was applied to the eyes as a topical anesthetic. An average of ten measurements was used to determine the IOP of the eye.

RETROGRADE LABELING OF RGCs

Retrograde labeling of RGCs was done in the 14 days groups immediately after the second laser treatment at the seventh day. Surviving RGCs were retrogradely labeled by applying a small piece of gelatin sponge (UpJohn, Kalamazoo, MI) soaked with Fluoro-Gold (FG) (Fluorochrome, Denver, CO) over the entire surface of bilateral superior colliculus (SC) as previously described [Ji et al., 2004; Chan et al., 2007; Chiu et al., 2008]. The RGCs of six rats from normal group were also labeled by the same method at 7 days prior to euthanization. After SC labeling, analgesic, bupreorphine (100 mg/kg), was orally administered for 5 days to relieve pain caused by the operation.

COUNTING OF RGCS AND STATISTICAL ANALYSIS

At 7 days after the SC labeling, after taking the IOP, six rats each from normal, LBP and PBS-fed OH 14 days groups were sacrificed with an over-dose of a mixture of ketamine/xylazine, and were perfused with 4% paraformaldehyde in PB (pH 7.4). Both eyes were enucleated and post-fixed in the same fixative for 60 min, then cut horizontally into superior and inferior eyecups. The superior eyecups with intact optic nerves were fixed overnight and processed to make paraffin blocks for further immunohistochemical study. Retinas from the inferior eyecups were dissected from the underlying sclera, and two radial cuts were made to divide the retina into three (nasal, inferior, and temporal) quadrants. The dissected retinas were then flattened with the vitreal side up and mounted using fluorescent mounting medium (Dako Corporation, Carpentaria, CA). The FG-labeled RGCs (FG particles in the cytoplasm) were visualized at 40× magnification using a fluorescent microscope with a UV-385 filter (Nikon, Kawasaki, Japan). The photos of RGC were taken ($200 \times 200\ \mu m^2$/microscope field) at 500 μm along the median line of each quadrant (8 microscopic fields/quadrant) starting from the optic disc to the peripheral border of the retina. After counting of the RGCs with the aid of a computer software developed by us (manuscript in preparation), the results were manually double checked by a person who is blind to the grouping. The average density of RGCs was calculated for the entire retina.

Changes in the density of RGCs were expressed as a percentage loss of RGCs by comparing the laser treated right eye and normal control eye [Panagis et al., 2005]:

$$\left[\frac{\text{RGC density in the normal eye} - \text{RGC density in the right eye with OH}}{\text{RGC density in the normal eye}}\right] \times 100\%$$

The percentage loss of RGC in different treatment groups was compared using one-way analysis-of-variance (ANOVA) followed by a post hoc Tukey multiple comparison test (SigmaStat, Statistical significance is noted as $^{*}P < 0.05$).

Two-dimensional gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization Time-of-Flight (MALDI-TOF) mass spectrometry (MS)/MS analyses.

PREPARATION OF RETINAL SAMPLES

To comprehensively examine different protein expression profiles among normal, LBP-fed, PBS-fed retina in rats, three time points was selected: normal rats with no treatment (normal, PBS-fed, and LBP-fed rat); 2-day post first laser (PBS-fed vs. LBP-fed) and 14-day post first laser (PBS-fed vs. LBP-fed). Immediately after the animal euthanization, the eyes were enucleated and then cornea and lens were carefully removed without destroying the lens capsule. Finally, the retinas were dissected from the underlying sclera, snapped frozen in liquid nitrogen. Both retinas from the right eyes with OH and the left eyes without OH were collected and stored at −80°C. Each frozen retina was lysed by homogenization in 200 μl Sequential Extraction Reagent 3 lysis buffer containing 5 M Urea,

2 M Thiourea, 2% (w/v) CHAPS, 2% (w/v) SB3-10, 40 mM Tris, 0.2% Bio-Lyte 3/10 (Bio-Rad, Hercules, CA), containing protease inhibitor cocktail (Sigma). Followed by incubation on ice for 30 min, the homogenates were centrifuged at 14,000 g for 30 min at 4°C to remove the debris and supernatants were then stored at −80°C until used for 2D-PAGE and Western-blot analysis. The protein concentration of all samples was determined by Bradford's method using the Bio-Rad protein assay.

TWO DIMENSIONAL SDS–PAGE AND IMAGE ANALYSIS

The procedures of 2D-PAGE and MS are similar to our previous published methods [He et al., 2003; Qi et al., 2008]. In each treatment group, two dimensional SDS–PAGE gels were done for four samples. To verify the consistency of individual changes in the protein level, three individual retinal samples were analyzed. One pooled retinal sample from the other three rats in the same treatment group was also tested to confirm the overall changes in protein levels. Briefly, 100 μg of total retinal proteins were mixed with rehydration solution containing 8 M urea, 4% CHAPS, 1 mM PMSF, 20 mM DTT and 0.5% IPG buffer to final 340 μl. The 13 cm IPG strips (pH 3 − 10 NL) were rehydrated for 12 h with low voltage of 30 V and followed by 500 V and 1,000 V for 1 h each and followed by 64 KVh. Next, the strips were subjected to two-steps equilibration in equilibration buffers containing 6 M urea, 30% glycerol, 2% SDS and 50 mM Tris–HCl (pH 6.8) with 1% DTT w/v for the first step, and 2.5% w/v iodoacetamide for the second step. Then the proteins were separated by 12.5% resolving gel for 4.5 h at 30 V per gel at room temperature. The gels were stained by Silver Staining. Briefly, the gels were fixed overnight in 40% ethanol and 10% acetic acid in water and then incubated in a sensitizing solution (30% ethanol, 4.1% sodium acetate and 0.2% sodium thiosulfate) for 30 min. Following three times washing with distilled water, the gels were stained in 0.1% silver nitrate solution containing 0.02% formaldehyde for 40 min. Then they were developed in a solution (containing 2.5% sodium carbonate and 0.01% formaldehyde) for 15 min and finally stopped by EDTA solution (1.46%). After three times washing in distilled water, Image acquisition and analyses were accomplished with ImageScanner (Labscan 3.00, GE Medical Systems) and ImageMaster (2-D Elite software 4.01, GE Medical Systems), respectively. Normalized volume for each protein spot was used for comparison. Only those spots changed significantly and consistently among different treatments were considered as interested spots to be picked up for MS and MS/MS analysis. All samples were run at least in duplicate to guarantee reproducibility.

IN-GEL TRYPTIC DIGESTION AND PROTEIN IDENTIFICATION

Spots of interest were excised and transferred to 1.5 ml low retention Eppendorf tubes. Gel chips were destained in a freshly prepared 1:1 mixture solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate and then equilibrated in 50 mM ammonium bicarbonate until pH 8.0 was reached. After dehydrating with acetonitrile and drying in a SpeedVac, the gels were rehydrated in a minimal volume of trypsin solution (10 μg/ml in 25 mM NH_4HCO_3) and incubated overnight at 37°C. The digests were then spotted onto a sample plate and coated with matrix CHCA (-cyano-4-hydroxycinnamic acid) (Sigma). MALDI-TOF MS/MS analyses were preformed on a ABI 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA). For the MS setting, reflector positive ion mode were used and scan range between 900 and 4,000 Da; For MS/MS setting, 2 KV positive with CID on and 5 monoisotopic precursors ($S/N > 200$) were selected. Calibration mixture 1, 4700 Proteomics Analyzer calibration mixture (from Applied Biosystems) was used as internal calibration standards. Protein identification was performed by searching NCBInr protein database (www.matrix-science.com) using automated GPS Explorer 3.6 software (Applied Biosystems) with the following criteria: (i) a maximum number of one missed cleavage; (ii) variable modifications were stated to be monoisotopic; (iii) carbamidomethyl (C) or Oxidation (M) were selected as a fixed modification and partial oxidation of methionine residues; (iv) mass tolerance was kept at 100 ppm; and (v) species searching was restricted to Rattus Norvegicus. The significant level of the results referring to the CI% was shown in the Table II.

WESTERN BLOT OF βB2-CRYSTALLIN

Thirty micrograms of aforementioned retinal protein samples from each treatment groups (3 from individual retina and 1 from pooled retinal samples) were separated by electrophoresis (Mini-Protean II system; Bio-Rad) on 12.5% resolving gel with 4% stacking gel. The electrophoresis was run at 60 V, 135 mA in electrophoresis buffer. Separated proteins on the gel were transferred to PVDF membranes (Bio-Rad) using the Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell at 10 V, 110 mA at room temperature for 2 h in transfer buffer. A blocking buffer containing 0.3% of normal serum in TBST (0.02 M Tris-buffered saline containing 1% Tween-20) was incubated with the membrane for 1 h. After washing with TBS, goat βB2-crystallin (N-20) primary antibody (Santa Cruz, CA) at 1:1,000 dilution was applied at room temperature for 2 h and followed by incubation with HRP-conjugated rabbit anti-goat secondary antibody (DAKO, Glostrup, Denmark) for 1 h. After thorough washing,

TABLE II. Identification of Proteins Differentially Expressed in Retinas

Spot #	Protein ID	MWt (KD)/PI	Accession #NCBI	Protein score/CI%	Total Ion score/CI%	Peptide count	Rank type
1	αA-Crystallin	23/6.5	gi130794510	337/100	85/100	11	Mascot
2	αB-Crystallin	20/6.64	gi57580	209/100	81/100	5	Mascot
3	βA4-Crystallin	22/5.9	gi13928956	168/100	81/100	4	Mascot
4	βB2-Crystallin	23/6.5	gi11598556	456/100	92/100	13	Mascot
5	βB2-Crystallin	23/6.5	gi11598556	145/100	97/100	10	Mascot

MWt, molecular weight; PI, isoelectric point.
Protein identification was performed by searching NCBInr protein database (www.matrix-science.com) using automated GPS Explorer 3.6 software.

bands were visualized on Biomax X-ray films (Kodak, Tokyo, Japan) using the Amersham ECL kit (GE Medical Systems). For internal loading control, the membrane was stripped with the Re-Blot Western blot recycling kit (Chemicon, Temecula, CA), and then re-probed for α-tubulin primary antibody (Sigma–Aldrich, St. Louis, MO) according to the aforementioned steps.

IMMUNOHISTOCHEMISTRY OF βB2-CRYSTALLIN

Retinal sections from different groups were handled at the same time for the primary antibody to avoid bench-to-bench variation. Four-micrometer retinal sections with intact optic nerves were used to detect the immunoreactivity of βB2-crystallin. The sections were deparaffinized and boiled in citric acid buffer (0.01 M, pH 6.0, 15 min). Following washing and blocking, retinal sections were incubated with βB2-crystallin (1:100) overnight at 4°C. After further washing, retinal sections were incubated with Alexa-594 fluorescent-conjugated secondary antibody (Molecular Probe) at room temperature for 1 h. The retinal sections were mounted and photos were taken under fluorescent microscope (Carl Zeiss, Jena, Germany) using identical exposure parameters. The specificity of antibody was tested by omission of the primary antibody.

RESULTS

Daily feeding of LBP started from 7 days prior to induction of ocular hypertension (OH) has been shown to be neuroprotective to retinal ganglion cells (RGC) under OH [Chan et al., 2007]. At 2 weeks after the first laser, loss of RGC was significantly decreased from 17.74 ± 1.07% (PBS group) to 1.01 ± 1.56% (LBP group). This neuroprotective effect was not through reduction of intraocular pressure. The baseline IOP before induction of OH in the right eyes that after 7 days feeding was 13.3 ± 0.7 mm Hg in PBS group control eyes, 13.2 ± 1.0 mm Hg in the LBP group eyes. At 2-day after the first laser, the IOP level in the right eyes with OH was significantly increased both in PBS control (26.3 ± 1.2 mm Hg) and LBP group (27.2 ± 1.1 mm Hg). At 2 weeks after the first laser, the IOPs in the OH eye were sustained at 24.5 ± 0.8 mm Hg (PBS) and 23.6 ± 0.8 mm Hg (LBP). There was no statistic difference of IOP between the PBS and LBP feeding.

Neuroprotective effect of LBP on RGC survival may involve various proteins, proteomic analysis was performed to give global analysis on protein expression. 2D-PAGE effectively separated most proteins into individual spots. The protein spots can then be visualized with silver staining, and the amount of protein on the gel is proportional to the silver intensity. Changes of protein profiles and spot intensities were compared at 2-day, 14-day post the first laser (PBS-fed vs. LBP-fed) and normal rats with no treatment (normal, PBS-fed and LBP-fed rat). Dramatic changes in retinal protein expression between the LBP treatment (Fig. 2B) and the PBS control (Fig. 2A) were detected at early time point (2-day post the first laser). Figure 2A′,B′ focused on a group of 5 spots, and the normalized spot volumes were shown in Figure 2C. Compared with PBS control, LBP induced 16.7, 10.2, 13.3, and 17.6 times increase in the volume of spot #1, 2, 4, and 5 respectively. Spot #3 was undetectable in PBS control, but can be detected in LBP treatment at 0.2 normalized volumes. These five spots that changed significantly and consistently among all four samples (including three individual and one pooled retinas) were picked up for protein identification. Table II summarized the identity of proteins with theoretical value of molecular weight (MWt) and isoelectric point (pI), protein score/CI% (which test the accuracy of match peptide fingerprints), total ion score/CI% (which test the accuracy of peptide sequence) and accession number of particular protein in NCBI database. These five spots are from the same protein family named—crystalline. Spot 1–3 represented αA-, αB-, βA4-crystallin, respectively. Spot 4 and 5 represented βB2-crystallin.

Crystallins are structural protein of lens, there is possibility the expression caused by lens rupture contamination. To solve this doubt, retinal dissection was started after the removal of anterior segment (including cornea, iris, and lens). The posterior segment was left with only retina and sclera. Lens contamination did not appear to be significant in the present study, as indicated by no detection of crystallins in normal groups (Fig. 3), and relatively consistent and selective crystalline staining profiles (Figs. 2 and 5). Compared to normal eye (Fig. 3A,A′), there were no detectable changes in retinal crystallin profile in the stress-free eyes from either PBS (Fig. 3B,B′) or LBP (Fig. 3C,C′) feeding. The LBP induced increased expression of crystallin in OH eyes was confirmed by Western blot. At 2 days after the first laser, expression βB2-crystallin were markedly increased in LBP treated OH retinas (Fig. 4). However, when compared the normal retina (Fig. 5A), OH retinas in PBS control group (Fig. 5B) or OH retinas in LBP treatment group (Fig. 5C), there were no detectable changes of crystallin spots at 14-day after the first laser photocoagulation.

To identify what kind of cells in the retina expressed βB2-crystallin, immunohistochemical staining was performed on the retinal sections. In normal retina, there were scattered βB2-crystallin immunoreactive RGCs in the ganglion cell layer (GCL) and inner nuclear layer (INL). The intensity of the βB2-crystallin staining in the retina is very weak (Fig. 6A). In PBS control group, at both 2- (Fig. 6B) and 14-day (Fig. 6E) after the first laser photocoagulation, βB2-crystallin immunoreactivity can be detected in the nerve fiberlayer (NFL) and weakly in scattered cells in the GCL and INL. With LBP treatment, βB2-crystallin immunoreactivity was markedly increased in the cells in the GCL and INL (arrows) at both 2- (Fig. 6C) and 14-day (Fig. 6F) after the first laser.

DISCUSSION

L. barbarum polysaccharide (LBP) from wolfberry is neuroprotective to retinal ganglion cells (RGCs) under ocular hypertension (OH) [Chan et al., 2007]. Using proteomic analysis, supported by Western blot and immunohistochemical detection, this study demonstrates that LBP treatment upregulates the expression of crystallin in the retina under OH, neuroprotective effect of LBP on the survival of RGCs may involve up-regulation of neuronal βB2-crystallin expression.

Since Bhat and Nagineni [1989] first found the extralenticular expression of the αB-crystallin in the retina, heart, brain and other tissues, it is now known that crystallins are prominent proteins both

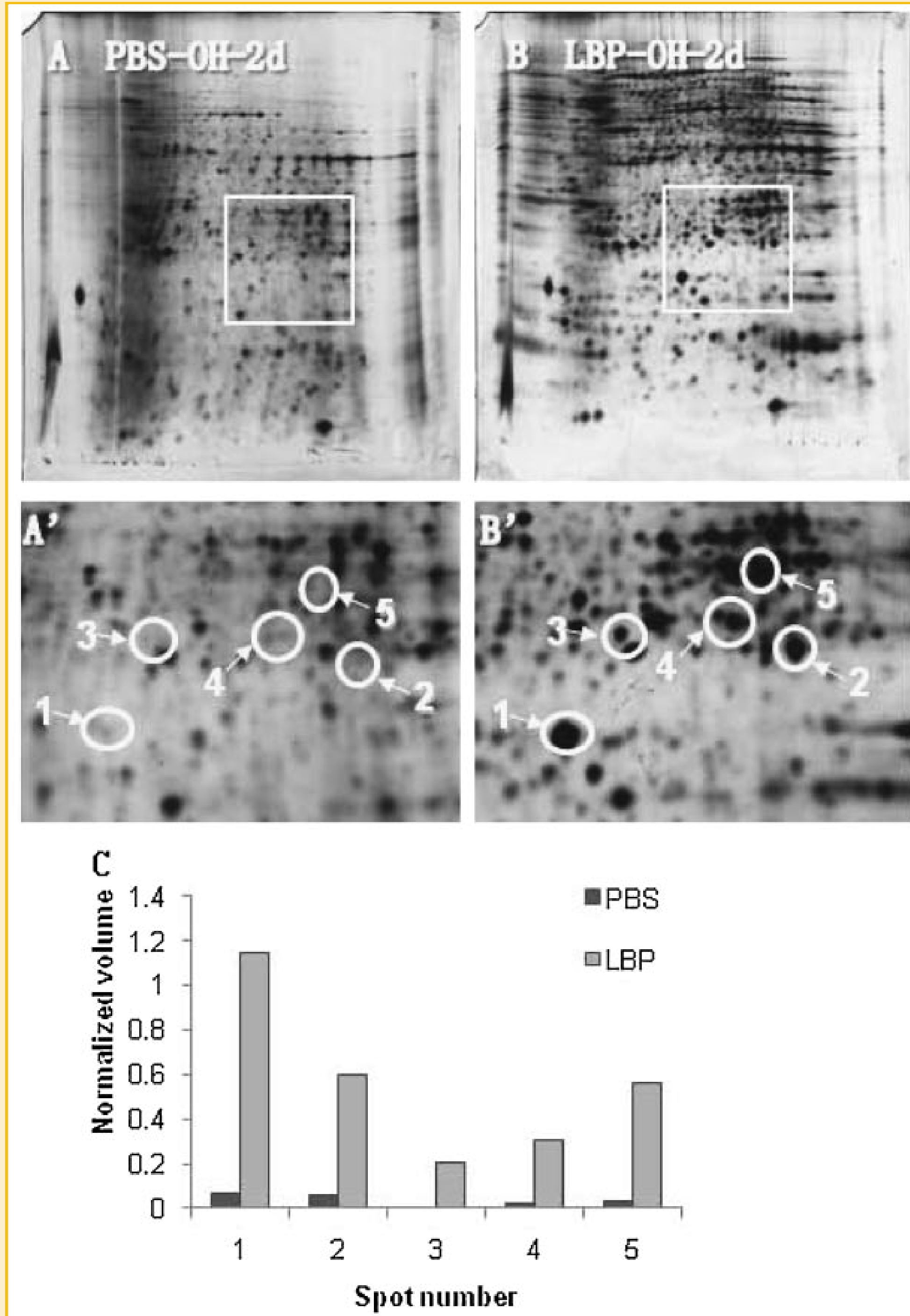

Fig. 2. 2D-PAGE of the retinal proteins at 2-day after the first laser photocoagulation. Proteins of whole retinal lysates were separated according to charge (IEF) and mass (SDS-PAGE). Focused 2D-PAGE areas were shown in the lower lane. Retinal protein profiles from OH retinas of the PBS-fed rats (A,A′) were shown in the left lane; and those from OH retinas of LBP-fed rats (B,B′) were shown in the right lane. Five spots identified that had marked changes between the two groups were labeled. These spots were cystallins as identified by MS/MS and their identifies were shown in Table II. The normalized volumes of the five spots were shown in (C). There was significantly increased in staining intensity in the OH eye of LBP group than the PBS group at 2-day after the first laser photocoagulation.

in normal retina [Head et al., 1991, 1995; Deretic et al., 1994; Jones et al., 1999; Magabo et al., 2000] and in retinal pathologies [Sakaguchi et al., 2003; Organisciak et al., 2006]. In situ result indicated that both alpha (αA and αB) and beta (βA2, βA4, and βB2) crystallin genes expressed in the RGCs of Wistar rat [Piri et al., 2007], and αA, αB and βH-crystallin immunoreactives were shown to be distributed in GCL, INL, etc. [Xi et al., 2003a,b]. Consistent to the previous study, the βB2 expression was in the GCL and INL of rat

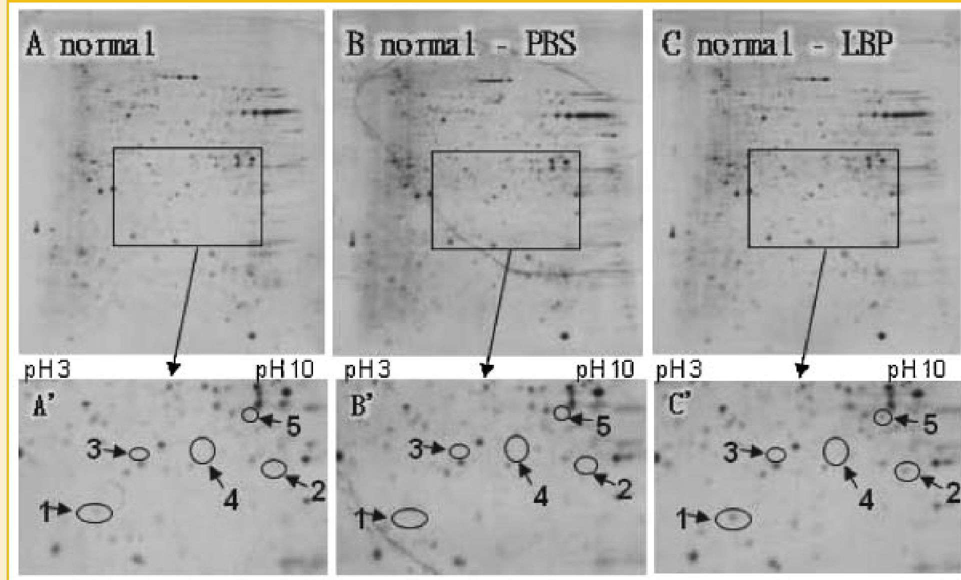

Fig. 3. 2D-PAGE of the retinal proteins from normal, normal PBS-fed and normal LBP-fed rats. Retinal lysates were from the normal (A), normal 7 days of PBS-fed (B), and normal 7 days of LBP-fed (C) rats. The expended gel areas (A′,B′,C′) of different samples correspond to boxed area in (A,B,C), and they were at the positions similar to the focused areas in Figure 2. Five spots identified as crystallins were also labeled.

retina. Lacking of positive staining of βB2 protein in the Western blot and trace staining spot in the proteomic analysis, indicated that crystallins are not abundant in SD rat retinas. Both oral feeding of PBS or LBP did not change the crystalline profiles in the non-stress retinas as shown in Figures 3 and 4.

At 2 days after the first laser, there were significant increases of both alpha (αA and αB) and beta (βA4 and βB2) in the OH retinas caused by LBP feeding. Comparing with PBS control, the normalized volume of both alpha (αA and αB) and beta (βA4 and βB2) crystallins were increased more than 10 times in LBP-treated rats as shown in Figure 2. Consistent to the findings that up-regulated expressions of crystallins in various retinal degenerations (genetic, age, light-mediated degeneration) demonstrated their important role in maintaining survival of retinal cell [Sakaguchi et al., 2003; Organisciak et al., 2006], this result indicated LBP-induced neuroprotection on the RGC survival may involve up regulated expression of retinal crystallins. There was persistent high levels of βB2 crystallin expression in the survival RGCs in the retina at 14 days after the first laser (Fig. 6F). However, LBP-induced up-regulation of crystallins could not be detected by proteomic at 14 days after the first laser (Fig. 5), indicating that cystallins might elicit the neuroprotection at early time.

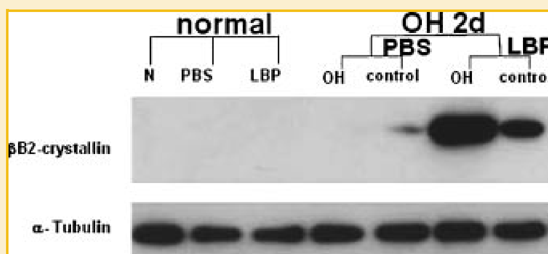

Fig. 4. Western-blot analysis of βB2-crystallin in retinas from the normal and 2-day after the first laser photocoagulation with PBS or LBP-fed. α-Tubulin was used as the internal control. Compared with PBS, LBP feeding significantly increased the βB2-crystallin expression in the OH eyes.

Our Western blot confirmed the LBP-induced up-regulation of βB2-crystallin in the OH eyes. To our surprise, compared with normal and the right OH eyes there was up-regulated βB2-crystallin expression in the left control eyes in the PBS treatment group (Fig. 4). There was no operation on the left control eyes in this study, why there was increased βB2-crystallin? Also, our data about the crystallin changes in experimental-induced OH retinas seemed to be different from Piri et al. [2007] reports. They showed decreased levels of alpha (αA and αB) and beta (βA2, βA4, and βB2) crystallins in the experimental eyes than the control eyes. We did not show how crystallins were affected by OH since they were barely detected in our system in the normal SD rat retinas. However, we detected up-regulated βB2-crystallin in the non-operated left control eye. If we used the protocol like Piri et al. by comparing the OH eye with the control eye, our conclusion would also indicate that OH caused down regulation of crystallins in the OH eye. There are increasing lines of evidence that unilateral nerve injury evokes contralateral responses [Bodeutsch et al., 1999; Kleinschnitz et al., 2005; Panagis et al., 2005]. Induction of OH in the right eyes may also cause stress in the contra-lateral left control eyes; and therefore, the use of the contralateral retina as a control in experimental glaucoma study is questionable.

In view of the direct cytoprotective and anti-aging effects of LBP, our previous work on Alzheimer's disease research has initiated a comprehensive investigation of LBP in counteracting β-amyloid peptide, glutamate and hyperhomocysteine toxicity in primary neuronal cell cultures [Yu et al., 2005, 2006, 2007; Ho et al., 2007,

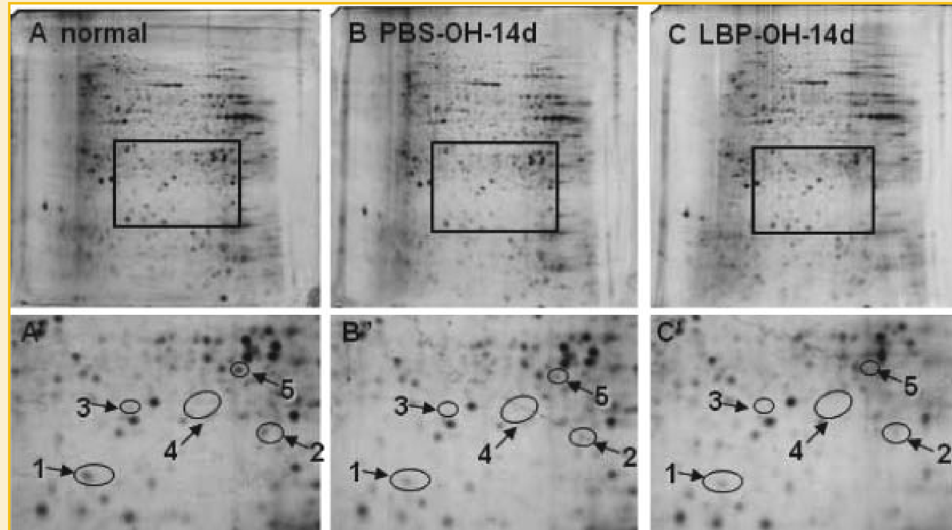

Fig. 5. 2D-PAGE of the retinal proteins at 2-day after the first laser photocoagulation. Retinal lysates protein profile from retinas of the normal rats (A,A′) was shown in the left lane; OH retinas of PBS-fed rats (B,B′) was shown in the middle lane; and those from OH retinas of LBP-fed rats (C,C′) were shown in the right lane. Compared to the normal retina, the five spots identified as crystallins did not show any significant changes in staining intensity in the OH retinas of both PBS and LBP-fed rats at 14-day after the first laser photocoagulation.

2009, 2010a]. In vitro, LBP prevents neuronal death via both necrosis and apoptosis with a wide range of effective dosages [Yu et al., 2006]. Although it has been shown that LBP elicits anti-oxidative effects [Wang et al., 2003; Li, 2007], LBP can inhibit two key pro-apoptotic signaling pathways (JNK and PKR) in Aβ peptide neurotoxicity independent of anti-oxidation property [Chang et al., 2002; Suen et al., 2003; Yu et al., 2005, 2006]. Recently, a new arabino-galactan-protein (LBP-III) isolated from LBP was reported

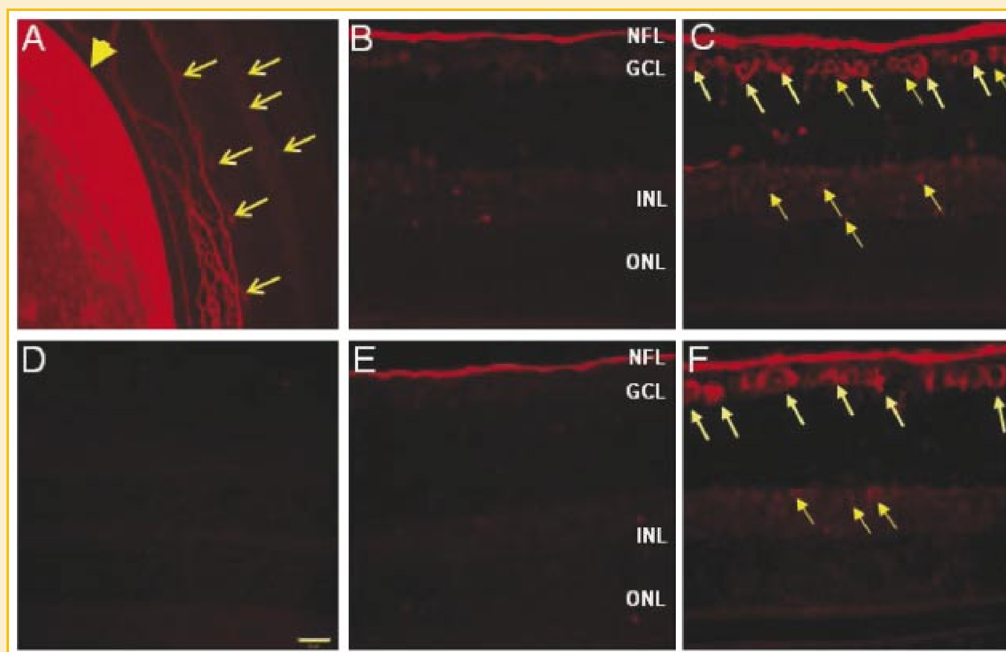

Fig. 6. βB2-Crystallin immunoreactivity in normal and the OH retinas from PBS and LBP-fed rats at 2-, 14-day after the first laser photocoagulation. In the normal rat eye, strong βB2-crystallin immunoreactivity was detected in the lens cortex (arrowhead, A). There were scattered βB2-crystallin positive cell in the GCL and INL (arrows, A). The intensity of the βB2-crystallin staining in the retina is very weak. In PBS control group, at both 2- (B) and 14-day (E) after the first laser photocoagulation, βB2-crystallin immunoreactivity can be detected in the NFL and weakly in scattered cells in the GCL and INL. With LBP treatment, βB2-crystallin immunoreactivity was markedly increased in the cells in the GCL and INL (arrows) at both 2- (C) and 14-day (F) after the first laser. No βB2-crystallin immunoreactivity was detected in the negative control (D). Scale bar is 20 μm for (B–F). Nerve fiber layer (NFL); ganglion cell layer (GCL); inner nuclear layer (INL); outer nuclear layer (ONL). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to attenuate the Aβ peptide-triggered caspase-3-like activity and the phosphorylation of PKR [Yu et al., 2007]. αA and αB-crystallins are involved in cell protection against stress inducible apoptosis by interacting with caspase 3, caspase 6 and Bax [Kamradt et al., 2001, 2002, 2005; Morozov and Wawrousek, 2006]. Therefore, pro-apoptotic signaling pathways including PKR, JNK and caspase-3-like activity should also be evaluated in the LBP neuroprotection against apoptotic RGCs deaths in experimental glaucoma.

To our knowledge, this is the first time demonstrating that βB2-crystallin may be involved in neuroprotection. It has been shown that axonal regeneration is related to crystalline βB2 movement [Liedtke et al., 2007]. Unlike other β-crystallins, βB2-crystallin has been shown to be heat stable like α-crystallin [Maiti et al., 1988]. The thermal stability and Ca^{2+} binding capacity of β-crystallins [Sharma et al., 1989] are both useful properties under stress conditions. Taken together, our study demonstrates that neuroprotective effects of Wolfberry involve up-regulation of βB2-crystallin. βB2-crystallin may be used as neuroprotective agents in treatment of neurodegenerative diseases like glaucoma.

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