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Protein profile of aging and its retardation by caloric restriction in neural retina

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Abstract

Aging is a slow, gradual deterioration process of an organism. The only experimental intervention, which can reliably retard aging and age-related degenerative diseases, is dietary caloric restriction (CR). To gain insight into the mechanism of CR intervention, we have investigated the protein profile of aging and its retardation by CR in the neural retina of Brown Norway (BN) rats using the comprehensive proteomic approach. We found that the intensities of 18 proteins decreased significantly with age. CR intervention can completely prevent seven of them, and partially protect eight of them, from such age-related declines. The major protein targets protected by CR intervention appear to be glycolytic enzymes and molecular chaperones. These data are the first to suggest that CR may retard the age-related degeneration of retina by maintaining sufficient glucose metabolism, by ensuring proper protein folding, and/or by preventing protein denaturation in the neural retina.

Keywords: Caloric restriction; Aging; Neural retina; Proteomics; Rat; Glycolytic enzymes; Molecular chaperones

Aging is a slow, gradual deterioration process of an organism. Retardation of aging is one of humankind's oldest dreams. Among the many attempts to retard the aging process, dietary caloric restriction (CR) is the only one that has been demonstrated through extensive experimentation to extend lifespan in a variety of species, including worms, flies, fishes, and rodents [1–4]. In most CR studies, the animals were fed 20–50% fewer calories than in usual feeding schedules (ad libitum fed, AL), but given all the necessary nutrients. Many studies have shown that CR also delays the occurrence of many age-related diseases such as cancers, nephropathy, cardiomyopathy, autoimmune diseases, cataract, and other degenerative eye diseases in rodents [4–8]. Now, a line of evidences further suggest that the CR intervention is also relevant to primates [9–11]. However, a critical question remains unanswered: by which mechanisms CR acts in mammalian animals.

To address such question, the neural retina is a suitable tissue. It consists of light sensitive photoreceptors and connecting neural cells that receive and send visual signal to the brain. Its function declines with aging, along with corresponding histological and biochemical changes [12,13]. This degeneration may contribute directly to the late-onset retinopathies in the elderly [14]. CR can reliably retard the age-related cell loss in the neural retina in a normal animal model, slow age pigment accumulation in rat retinal pigment epithelium, and reduce the risk of glaucoma [6–8]. Our recent studies further demonstrated that CR slowed the age-dependent protein insolubilization, blunted the declines in the total soluble thiols, and reduced glutathione, ascorbic acid, and taurine levels in neural retina [15]. To gain a broader, deeper insight into the mechanism of CR intervention, we have investigated the protein profile of aging and its retardation by CR in the neural retina of Brown Norway (BN) rats using the comprehensive proteomic approach.

Materials and methods

Animal rearing and handling. This study was approved by the Institutional Animal Care and Use Committee of Columbia University.

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All procedures adhered to the NIH guidelines for the use of animals in research. The male BN rats were members of the aging and caloric restriction colony raised in the pathogen-free barrier facility at Harlan Sprague Dawley (Indianapolis, IN). The ad libitum (AL) cohorts were fed the standard NIH-31 diet. The CR animals were fed a vitamin-enriched NIH-31 diet to bring vitamin levels to equivalence with the AL diet [16]. CR was initiated at 14 weeks of age at 10% restriction, changed to 25% restriction at 15 weeks, and to 40% restriction at 16 weeks where it was maintained throughout the life of the animal. The room was illuminated with white fluorescent lights on a 12/12 light cycle. A total of 26 male BN rats were used in this study. There were ten 28-month-old rats (five AL and five CR), ten 31-month-old rats (five AL and five CR), and six young rats at 2 months old. The rats were shipped in individual cage with food and water. The rats were euthanized with carbon dioxide inhalation and the eyes were dissected immediately.

Tissue preparations. The eyes were slid open with a sharp surgical knife. With the aid of dissecting microscope, the neural retina layer was carefully peeled off from the posterior section of the eye and placed on a weighing paper. After the removal of the adhering vitreous, the neural retina was weighed. No retinal pigment epithelium segment was found on these neural retina preparations. The neural retina from one eye was homogenized in 0.20 ml of 20 mM phosphate (pH 7.4) with a cocktail of protease inhibitors (Boehringer–Mannheim, Germany) at 4 °C under an argon atmosphere, and centrifuged at 10,000*g* for 10 min. The supernatants, defined as the water-soluble fraction, were stored in aliquots at −70 °C for later use.

Protein content measurement. The protein concentration in the water-soluble fraction of each preparation was determined with the Bio-Rad (Hercules, CA) dye protein assay kit using bovine serum albumin as the standard.

Two-dimensional gel electrophoresis and MALDI-MS peptide mass mapping. The water-soluble proteins (about 200 μg) from each retina preparation were applied to a Pharmacia 13-cm, pH 3–10, NL IPG strip, and isoelectric focused on a Pharmacia IPGphor System (Amersham–Pharmacia, Piscataway, NJ) about 10,000 V × h for each strip. The procedure has four steps: rehydration 12 h; 500 V, 1 h; 1000 V, 1 h; and 8000 V, 5 h. Then the strip was equilibrated in a SDS equilibration buffer, applied on the top of a 12.5% separating gel, and run the second dimension SDS–PAGE. The 2D gels were visualized with a modified silver stain procedure derived from previous methods ([17,18], Bio-Rad Silver Stain Plus Manual). The procedure involved eight steps: fixation, Bio-Rad Silver Stain Plus Fixative Enhancer Solution, 60 min; rinse, water 2 × 20 min; sensitization, sodium thiosulfate (0.2 g/L), 20 min; rinse, water, 2 × 2 min; silver, silver nitrate (1.0 g/L), 30 min; rinse, water, 2 min; development, 0.04% formaldehyde in 2.5% sodium carbonate, 2 min + 10 min or to desired intensity; stop, 5% acetic acid, 10 min; and store, water. This revised protocol yielded a low background gel and was suitable for matrix-assisted laser desorption ionization (MALDI) mass spectroscopic (MS) analysis. Five marker proteins from Bio-Rad were used to standardize the 2D-gel pattern: bovine serum albumin (66.2 kDa, p*I* 5.44), chicken egg ovalbumin (45.0 kDa, p*I* 4.96), bovine carbonic anhydrase (31.0 kDa, p*I* 6.46), soybean trypsin inhibitor (21.5 kDa, p*I* 4.42), and egg white lysozyme (14.4 kDa, p*I* 9.22). Quantitative differences of corresponding spots were analyzed with NIH Image software, using the unchanged protein spot 3 as the internal standard. Four samples from each group were used. The spots with significant changes between the young and the old, and between the CR and the AL rats were excised and sent to the Protein Core Facility at Columbia University for protein identification. In the facility, the protein in the gel was digested with trypsin, followed by MALDI-MS analysis of the resulting peptide mixture. The peptide masses obtained by MS were entered into a search program, which scans the database of NCBI or Genpept to find a match. The search programs used are ProFound at http://prowl.rockefeller.edu/cgi-bin/ProFound and MS-Fit at http://prospector.ucsf.edu.

Western blotting. The water-soluble proteins (about 5 μg) from neural retina homogenates were separated by SDS–PAGE on 12.5% gel and transferred to nitrocellulose membrane. The αB-crystallin band was detected by anti-αB-crystallin antibody (from Dr. Joseph Horwitz, UCLA) with an Amersham ECL Western blotting analysis system. The protein bands were quantitated with NIH Image software.

Results and discussion

The protein profile of aging and its retardation by CR in rat neural retina were studied with 2D-gel electrophoresis. The typical gels of the 2-month-old and the 31-month-old AL and CR male BN rats are shown in Fig. 1. As marked on the gel of the 2-month-old rat, three major protein spots 1, 2, and 3 remained similar intensities on all three gels.

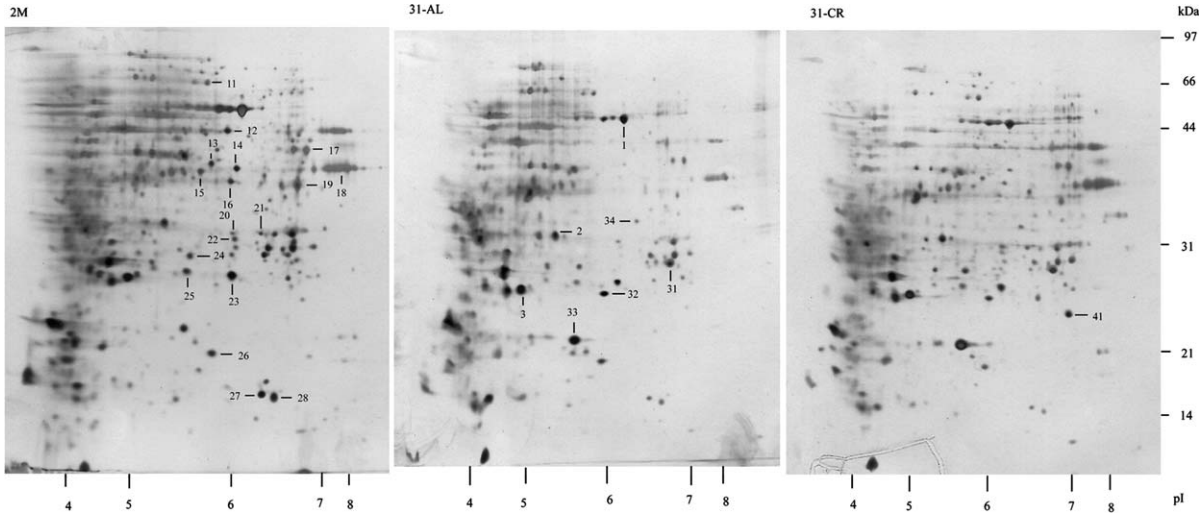

Fig. 1. The typical silver-stained 2D gel electrophoresis images of water-soluble proteins of the neural retinas from the young rat (2M) and the old rats on AL (31M-AL) or CR diet (31M-CR).

Proteins whose level decreased with age

The maximum life span of male BN rat is about 35 months on AL diet and is about 43 months on CR diet [16]. Comparing the gel of 31M-AL with that of the 2M sample, the intensities of many protein spots decreased significantly with age. Using the protein spot 3 as the internal standards, the intensities of 18 protein spots (11–28) decreased at least 50% at the advanced age.

Four of them were identified with MS/Peptide Mapping as glycolytic enzymes, phosphoglycerate kinase 1, glyceraldehyde 3-phosphate dehydrogenase, and two malate dehydrogenases (cytoplasmic and mitochondrial), as summarized in Table 1. Interestingly, CR intervention could completely prevent two of them, phosphoglycerate kinase 1 and malate dehydrogenase-mitochondrial, from age-related declines, and partially protect the other two. It is conceivable that these glycolytic enzymes may play an important role in delaying the age-related degeneration of the retina by providing the needed energy to maintain the normal retinal function in old CR animals. Similarly, CR could also retard the age-related decline of the genes involved in energy metabolism in skeletal muscle of mice [19].

Two proteins, whose levels decreased with age, were identified as endoplasmic reticulum chaperones, endoplasmic reticulum proteins 29 (ERP29), and protein disulfide isomerase A3 (PDIA). ERP29 is a 24.5 kDa soluble protein, postulated to function within a network of molecular chaperones and foldases that facili-

Table 1
Major age-related and CR-regulated changes on silver-stained 2D gels of neural retinas

Spot	Identification	Swiss-Prot	Possible function	Experimental Da, p*I*	Theoretical kDa, p*I*	Aging	CR
1	α-Enolase	P04764	Glycolysis	50, 6.2	47, 5.8	N	N
2	Asialoglycoprotein-binding protein	P49301	Immune response	33, 5.4	34, 5.2	N	N
3	Phosphatidylethanolamine-binding protein	P31044	Protease inhibitor	24, 5.0	21, 5.5	N	N
11	Protein disulfide isomerase A3	P11598	ER chaperone	58, 5.7	57, 5.9	D	C
12	Unidentified			44, 6.0		D	P
13	Unidentified			38, 5.7		D	P
14	Unidentified			37, 6.1		D	P
15	Unidentified			36, 5.5		D	P
16	Malate dehydrogenase, cytoplasmic	P14152	Glycolysis	35, 6.0	36, 6.2	D	P
17	Phosphoglycerate kinase 1	P09411	Glycolysis	42, 6.8	44, 7.5	D	C
18	Malate dehydrogenase, mitochondrial	P04636	Glycolysis	38, 7.5	36. 8.9	D	C
19	Glyceraldehyde 3-phosphate dehydrogenase	P04797	Glycolysis	35, 6.7	36, 8.4	D	P
20	Endoplasmic reticulum protein 29	P52555	ER chaperone	30, 6.1	26, 6.1	D	C
21	Aryl sulfotransferase	P17988	Drug and hormone metabolism	31, 6.4	34, 6.4	D	C
22	Hypoxanthine-guanine phosphoribosyltransferase	P27605	Purine salvage	29, 6.1	24, 6.1	D	C
23	Contraception-associated protein 1	O88767	Fertilization	25, 6.1	20, 6.3	D	P
24	Apolipoprotein A-I	Q00623	Lipid transporter	28, 5.5	30, 5.6	D	C
25	High mobility group protein 1	P07155	DNA binding	26, 6.2	25, 5.6	D	P
26	CuZn-SOD	P07632	Antioxidant enzyme	19, 6.0	16, 5.9	D	N
27	Unidentified			16, 6.4		D	N
28	Fatty acid-binding protein, epidermal	P55053	Signaling molecules	16, 6.6	15, 6.7	D	N
31	βB2-Crystallin	P26775	Ca^{2+} buffering?	25, 6.7	23, 6.5	I	N
32	Unidentified			24, 5.9		I	P
33	αA-Crystallin	P02490	Chaperone	21, 5.8	20, 5.8	I	N
34	3-Hydroxyisobutyrate dehydrogenase	P29266	Lipid metabolism	33, 6.3	32, 6.2	I	N
41	αB-Crystallin	P23928	Chaperone	23, 6.9	20, 6.8	I	A

I, increased; D, decreased; C, complete protection; P, partial protection; N, no significant effect; A, amplification. The results are the averages of four samples from each group.

tate folding, posttranslational modifications, and transport of the secretory proteins in endoplasmic reticulum [20]. ERP29 was induced to high levels in the rat hepatoma cells under metabolic stress conditions known to cause an aberrant accumulation of proteins in the ER [21]. PDIA is a 56.6 kDa soluble protein, contains two thioredoxin domains, and belongs to the protein disulfide isomerase family [22,23]. It may have a critical role in the rearrangement of both intrachain and interchain disulfide bonds in proteins to form the native structures. Thus, the age-related declines of these ER chaperones may cause improper protein folding in the neural retinas of old animals. Interestingly, CR intervention could completely prevent these two ER chaperones from age-related decline. Therefore, the higher levels of these two ER chaperones in the neural retinas of CR rats might contribute directly to CR's beneficial effect in delaying age-related degeneration of the retina.

Other proteins, whose levels also decreased with age, were identified as aryl sulfotransferase (AST), hypoxanthine-guanine phosphoribosyltransferase (HPRT), apolipoprotein A-I (ALPA), contraception-associated protein 1 (CAP1), high mobility group protein 1 (HMRP1), CuZn-SOD, and fatty acid-binding protein (FABP). CR intervention completely prevented three of them, AST, HGPT, and ALPA, from age-related decline. AST belongs to the sulfotransferase family, which catalyzes the transfer of sulfate (sulfonation) from the physiological sulfate donor 3′-phosphosulfate-5′-phosphoadenosine to the appropriate substrate or metabolite [24,25]. It is a key component of drug and hormone metabolism and widely expressed in hepatic and extrahepatic tissues. It is critical for the detoxication and elimination of toxic intermediates. HPRT is a cytosolic protein, belonging to the purine/pyrimidine phosphoribosyltransferase family. It is probably involved in purine salvage [26,27]. HPRT deficiency is an X-linked disorder and the pathologies associated with partial or virtually complete deficiency of this enzyme range from hyperuricemia, uric acid stones, and gout to severe neurological traits. ALPA belongs to the apolipoprotein A1/A4/E family, participating in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase. It displayed remarkable atheroprotective activities [28,29]. Thus, these proteins may also play an important role in delaying the age-related degeneration of the retina in old CR animals.

Proteins whose level increased with age

As marked in Fig. 1, age-dependent increases were also observed on four protein spots (31–34), however, CR seemed to have little effect in preventing these increases. Three of increased proteins were identified as αA-crystallin, βB2-crystallin, and 3-hydroxyisobutyrate dehydrogenase (HBD). The higher expression of αA-crystallin may be retina's adjustment to higher levels of unfolded proteins in the retinas associated with aging, since αA-crystallin can act as a molecular chaperone [30]. The role of βB2-crystallin in the retina is obscure, possibly as a Ca^{2+} buffer for maintaining calcium homeostasis in the retina [31]. Increased expression of crystallins had also been reported in the light exposed rat retina [32]. HBD is a metabolic enzyme for lipid [33]. Higher level of HBD may reflect the increased need for energy because of the declined glucose metabolism in the neural retinas of old animals.

Stress protein αB-crystallin

One striking observation was a strong protein spot 41 on the 31M-CR gel, which was a weak spot on the 31M-AL gel, and undetectable on the 2M gel. This spot matched exactly with the location of αB-crystallin prepared from rat lens on the gel. Indeed, the identity of this protein was identified by peptide mapping as αB-crystallin, and further confirmed by Western blot analysis as shown in Fig. 2. Densitometric analysis of the blot indicates that αB-crystallin was highly expressed in the neural retinas of the 31-month and 28-month-old CR rats, but was only moderately expressed in those of the 31-month-, 28-month-old AL rats and the 2-month-old rats. Since the plasma glucocorticoid level increased with age as well as with CR treatment in rats and since glucocorticoids could stimulate the expression of αB-crystallin in the cell, a possible CR induced high level of glucocorticoid in neural retina may lead to high expression of αB-crystallin [34,35].

αB-Crystallin is a stress protein and highly expressed in mammalian lenses. It can act as a molecular chaperone [36,37]. It could protect other proteins from stress induced aggregation, safeguard the cytoskeleton structure, mediate an increase in GSH level, and inhibit apoptotic cell death. The presence of αB-crystallin in the retina had first been observed by Deretic et al. [38]. The newly synthesized α-crystallins (include αA and αB) associated with the post-Golgi membranes concurrently

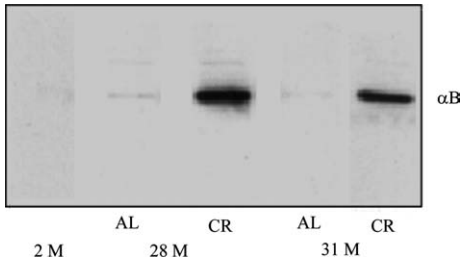

Fig. 2. Western blot analysis of αB-crystallin in the extracts of the neural retinas.

with newly synthesized rhodopsin suggest that they may participate in photoreceptor outer membrane renewal. The expression of αB-crystallin could be stimulated by hyperthermia and light exposure [32,39]. Furthermore, hyperthermia pretreatment could protect rat retina against later light damage [40]. Adding together, these data suggest that, as a molecular chaperone, the increased αB-crystallin may also play a role in the survival of the retinal cells in old CR animals.

In summary, our work suggests that CR may retard the age-related degeneration of retina by protecting glycolytic enzymes, by maintaining or stimulating the levels of molecular chaperones, and by sustaining the pool of other vital proteins such as AST, HGPT, and ALPA in the neural retina of old BN rats.

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