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Effect of chronic hyperglycemia on crystallin levels in rat lens

Vadde Sudhakar Reddy, Chekkilla Uday Kumar, Geereddy Bhanuprakash Reddy *

Biochemistry Division, National Institute of Nutrition, Hyderabad, India

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ABSTRACT

Crystallins are the major structural proteins in the vertebrate eye lens that contribute to lens transparency. Although cataract, including diabetic cataract, is thought to be a result of the accumulation of crystallins with various modifications, the effect of hyperglycemia on status of crystallin levels has not been investigated. This study evaluated the effect of chronic hyperglycemia on crystallin levels in diabetic cataractous rat lens. Diabetes was induced in rats by injecting streptozotocin and maintained on hyperglycemia for a period of 10 weeks. At the end, levels of α-, β-, γ-crystallins and phosphoforms of αB-crystallins (αBC) were analyzed by immunoblotting. Further, solubility of crystallins and phosphoforms of αBC was analyzed by detergent soluble assay. Chronic diabetes significantly decreased the protein levels of α-, β- and αA-crystallins (αAC) in both soluble and insoluble fraction of lens. Whereas γ-crystallin levels were decreased and αBC levels were increased in lens soluble fraction with no change in insoluble fraction in diabetic rat lens. Although, diabetes activated the p38MAPK signaling cascade by increasing the p-p38MAPK in lens, the phosphoforms of αBC were decreased in soluble fraction with a concomitant increase in insoluble fraction of diabetic lens when compared to the controls. Moreover, diabetes strongly enhances the degradation of crystallins and phosphoforms of αBC in lens. Taken together, the decreased levels of crystallins and insolubilization of phosphoforms of αBC under chronic hyperglycemia could be one of the underlying factors in the development of diabetic cataract.

1. Introduction

Diabetes and its complications have emerged as a major public health concern worldwide as global prevalence of diabetes is rising at an alarming rate. It is estimated that currently around 382 million people have diabetes globally and the number is projected to rise to 592 million in 2030 [1]. Chronic uncontrolled or poorly controlled diabetes can lead to micro and macrovascular complications [2]. Blindness due to cataract is an important long-term complication of diabetes. Cataract, characterized by cloudiness or opacification of the eye lens, is the leading cause of blindness all over the world and more so in the developing countries [3]. The prevalence of diabetic cataract is steadily rising due to increase in number of people with diabetes all over the world. Studies indicate that the incidence of cataract is much higher in diabetic than in non-diabetic individuals [3,4]. Though the etiology of cataract is not fully understood, oxidative damage to the constituents of the eye lens is considered to be the major mechanism in the initiation and progression of various types of cataracts, including diabetic cataract [5].

Crystallins are the major structural proteins in the vertebrate eye lens that account for about 90% of the total soluble protein [6]. There are three major crystallins, α, β and γ- belonging to two protein families. The two α-crystallins, αA- and αB-crystallin (αAC and αBC) that are members of the family of small heat shock proteins (sHsp), constitute ~30% of lens proteins and occur as hetero oligomeric complexes of variable size, ranging from 300 kDa to over 800 kDa with each monomer being ~20 kDa [7,8]. There are a large number of β- and γ-crystallins which share the same core tertiary structure and are often referred to as the β/γ-family, which includes a diverse group of non-lens proteins with a similar tertiary structure. Structure, stability and short-range interactions of crystallins are thought to contribute to the lens transparency [9]. α-Crystallins exhibit chaperone like activity (CLA) defined by their ability to bind partially unfolded proteins and thereby prevent their aggregation. The CLA of α-crystallins is shown to be declined in many experimental and human cataracts including diabetic cataract [8,10]. Members of the β/γ-superfamily, which include β-crystallins (βA1/A3, βA2, βA4, βB1, βB2 and βB3) and γ-crystallins (γA–F, and γS), are induced by physiological stress and are related to microbial proteins [11].

* Corresponding author. Address: National Institute of Nutrition, Hyderabad 500 007, India. Fax: +91 40 27019074.
E-mail address: geereddy@yahoo.com (G.B. Reddy).

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The long-term complications of diabetes, including diabetic cataract, are thought to be a result of the accumulation of tissue macromolecules that have been progressively modified by various posttranslational modifications (PTM). Being long lived proteins with slow turnover, crystallins are known to undergo extensive PTM including oxidation, mixed disulphide formation, deamidation, racemization, truncation, phosphorylation and glycation [6,12]. Most of these PTM have been shown to occur with aging and are accelerated in clinical conditions such as diabetes. Among them, non-enzymatic glycation has been considered to be one of the mechanisms responsible for both age-related and diabetic cataracts [13,14]. Previously, we have described the effect of glycation on the structure and CLA of α-crystallin and its contribution to diabetic cataract formation [10,15,16]. We have also reported the elevated expression of α-crystallins in various tissues including lens in diabetic rats [17] and also response of all sHsp family members in diabetic rat retina [18]. However, the effect of chronic hyperglycemia on the levels of crystallins and response of sHsp in lens has not been investigated. The phosphorylation status of α-crystallins is an important factor in modulation of various functions including CLA. αBC is phosphorylated at three sites Serine-59 (S59), S45, S19. While the S59 of αBC is phosphorylated by p38–mitogen-activated protein kinase (p38MAPK), S45 is phosphorylated by ERK [19]. The kinase responsible for phosphorylation of S19 of αBC is unknown. However, the effect of diabetes on kinase mediated phosphoregulation of αBC in lens is not known. Hence, in this study we report the levels of α-, β-, γ-crystallins, and phosphorylation status of αBC under chronic hyperglycemic conditions in rat lens.

2. Materials and methods

2.1. Materials

Streptozotocin (STZ), Tri-reagent, TritonX-100 (TritonX), acrylamide, bis-acrylamide, ammonium persulphate, β-mercaptoethanol, SDS, TEMED, PMSF, aprotinin, leupeptin, pepstatin, anti-actin antibody, horse radish peroxidase (HRP) conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Sigma Chemicals (St. Louis, MO, USA). Nitrocellulose membrane was obtained from Pall Corporation (Pensacola, FL, USA). Anti-α, β, γ-crystallin antibodies were generously gifted by Dr. Samuel Ziegler (Johns Hopkins University). Anti-αAC and anti-αBC antibodies were produced in rabbit as reported earlier [20]. Specific antibodies recognizing three phosphorylated residues (S59, S45, and S19) of αBC were obtained from Thermo Scientific; Pierce (Rockford, IL, USA). Anti-p38MAPK, anti-p-p38MAPK antibodies were purchased from Cell Signaling Technology. Anti-GRIFIN antibody was obtained from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) kit was obtained from GE Health Care (Buckinghamshire, UK).

2.2. Animal care and experimental conditions

Three-month old male Wistar–NIN rats with average body weight of 223 ± 14 g were obtained from National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad and maintained at a temperature of 22 ± 2 °C, 50% humidity and 12 h light/dark cycle. The control rats ($n = 9$) received 0.1 M sodium citrate buffer, pH 4.5 as a vehicle while the experimental rats ($n = 9$) received a single intraperitoneal injection of STZ (35 mg/kg) in the same buffer. After 72 h of STZ injection, fasting blood glucose levels were monitored and animals with blood glucose levels >150 mg/dl were considered for the experiment. Both control and diabetic animals were fed with AIN-93 diet *ad libitum*. At the end of 10 weeks, rats were fasted overnight and sacrificed by CO_2 asphyxiation. Animal care and protocols were in accordance with and approved by Institutional Animal Ethics Committee (IAEC). The 10-weeks diabetes duration was chosen because by then all the animals developed mature cataract.

2.3. Slit lamp examination and lens collection

Eyes were examined for lens opacity using a slit lamp biomicroscope (Kowa SL15, Portable slit lamp, Tokyo, Japan). Initiation and progression of lenticular opacity was graded into five categories as described previously [21]. The eye balls were collected and lenses were dissected.

2.4. Whole tissue lysate preparation

Lenses were homogenized in TNE buffer (0.02 M Tris buffer, pH 7.5 containing 0.1 M NaCl, 0.001 M EDTA, 0.001 M DTT and protease inhibitors). Homogenization of lens was performed on ice using a glass homogenizer and the homogenate was centrifuged at 12,000*g* at 4 °C for 20 min to obtain total soluble fraction. The protein concentrations were measured by Bradford method.

2.5. SDS–PAGE and immunoblotting

Equal amounts of protein (40 μg for α-, β- and γ-crystallins, 25 μg for αAC, αBC, and GRIFIN, 50 μg for pS59, pS45 and pS19 of αBC) from control and diabetic lens was subjected to 12% SDS–PAGE. The proteins were transferred onto nitrocellulose membranes and incubated overnight at 4 °C with respective primary antibodies diluted in PBST (20 mM phosphate buffer, pH 7.4; 137 mM NaCl, 0.1% Tween-20), separately. After washing with PBST, membranes were incubated with anti-rabbit IgG or anti-mouse IgG secondary antibodies conjugated to HRP. The immunoblots were developed with ECL detection reagents by using Image analyzer (G-Box iChemi XR, Syngene, UK) and images were quantitated using the Image J software (available at http://rsb-web.nih.gov/ij/).

2.6. Detergent solubility analysis

Lenses were homogenized in TNE buffer containing 0.5% TritonX. Following centrifugation, supernatant containing the detergent-soluble fraction and pellet containing detergent-insoluble protein fractions were separated. The pellet was washed with PBS, rehomogenized, sonicated and dissolved in Lammelli buffer. These samples were analyzed by immunoblotting as described above.

2.7. Co-immunoprecipitation (Co-IP)

Co-immunoprecipitation was carried out using Co-IP kit (Thermo scientific; Pierce, Rockford, IL, USA) according to the manufacturer instructions. Briefly, the lenses were homogenized in IP lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4) and protein concentrations were measured using Bradford method. Lens homogenate containing 1 mg protein was precleaned using 80 μl control agarose resin slurry and centrifuged at 1000*g* for 1 min. The precleaned homogenate was added to spin column with immobilized αAC antibody and incubated at 4 °C overnight with gentle mixing. The spin column was centrifuged and washed 3 times with 200 μl IP lysis/wash buffer and eluted with elution buffer. The eluate was loaded onto SDS–PAGE, followed by immunoblotting using anti-GRIFIN, anti-αAC and anti-αBC antibodies.