Biochemical and Biophysical Research Communications 446 (2014) 602–607

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effect of chronic hyperglycemia on crystallin levels in rat lens

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ARTICLE INFO

Article history:
Received 25 February 2014
Available online 12 March 2014

Keywords:
Cataract
Crystallins
Diabetes
Phosphorylation
p38MAPK
Rat lens

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].

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http://dx.doi.org/10.1016/j.bbrc.2014.03.012
0006-291X/

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.

2.8. Statistical analysis

Student's *t*-test was used for the comparison between two groups. Data are presented as mean ± S.E and expressed as percent of control.

3. Results and discussion

3.1. Body weight, blood glucose, HbA1c levels and protein content

As reported earlier [18], STZ-treated experimental diabetic rats demonstrated elevated fasting blood glucose, HbA1c levels and decreased body weights when compared to controls. There was a significant decrease in both total protein (489 ± 9.96 vs. 332 ± 10.7 mg/g lens; $p < 0.001$) and total soluble lens protein (359 ± 10.2 vs. 151 ± 10.0 mg/g lens; $p < 0.001$) in diabetic rats compared with the control rats.

3.2. Cataract progression

The onset of cataract was observed in diabetic animals after 4 weeks of STZ injection due to hyperglycemia and progressed to mature cataract by 10 weeks. All the lenses of control group appeared normal and free of opacity on slit-lamp examination.

3.3. Crystallin levels in diabetic cataractous lens

We have assessed the levels of three major crystallins in lens by immunoblotting with antibodies directed against total α-, β-, γ-crystallins. The levels of total α-crystallin were significantly decreased in diabetic rat lens when compared with controls (Fig. 1A and B). Immunoblot analysis of total α-crystallin revealed three bands in control corresponding to *αAC*, *αBC* and *αAC-insert* whereas multiple bands were observed in diabetic lens which could be due to truncation and/or degradation in diabetic conditions. Similarly, β- and γ-crystallins were also significantly decreased in diabetic animals when compared to controls (Fig. 1A and B). Together, these results indicate that all the three major crystallins, α, β, and γ, were decreased in total soluble fraction of diabetic rat lens. α-Crystallin consists of αAC and αBC sub units present in a 3:1 M ratio in vertebrate lenses [22–24]. Further, αAC- and αBC are reported to behave differently with respect to chaperone activity and structural transitions under different conditions [8,23,25]. Therefore effect of diabetes on αAC and αBC was analyzed using αAC and αBC specific antibodies (Fig. 1C). It is interesting to note that αAC protein levels were significantly decreased whereas αBC was significantly increased (Fig. 1C and D). Further, multiple bands with considerable degradation of αAC was observed in diabetic animals in comparison with controls. Though, αBC levels were increased in diabetes, the decrease in total α-crystallin could be due to decreased αAC which is in predominant proportions in α-crystallin.

3.4. Kinase mediated phosphoregulation of αBC in diabetic cataract

αBC functions are modulated by phosphorylation. Therefore, we examined the phosphorylation status of αBC with specific antibodies recognizing the three phosphorylated residues at these specific sites – S59, S45, and S19. All these phosphorylated forms of αBC were abundantly detected in lens. Interestingly, while the phosphorylation at S45 and S19 of αBC was unaltered, phosphorylation at S59 of αBC was decreased in total soluble fraction of diabetic lens when compared to controls (Fig. 2A and B). Immunoblot analysis of pS45 and pS19 of αBC revealed two bands in control and multiple bands in lens of diabetic animals. The multiple bands could have

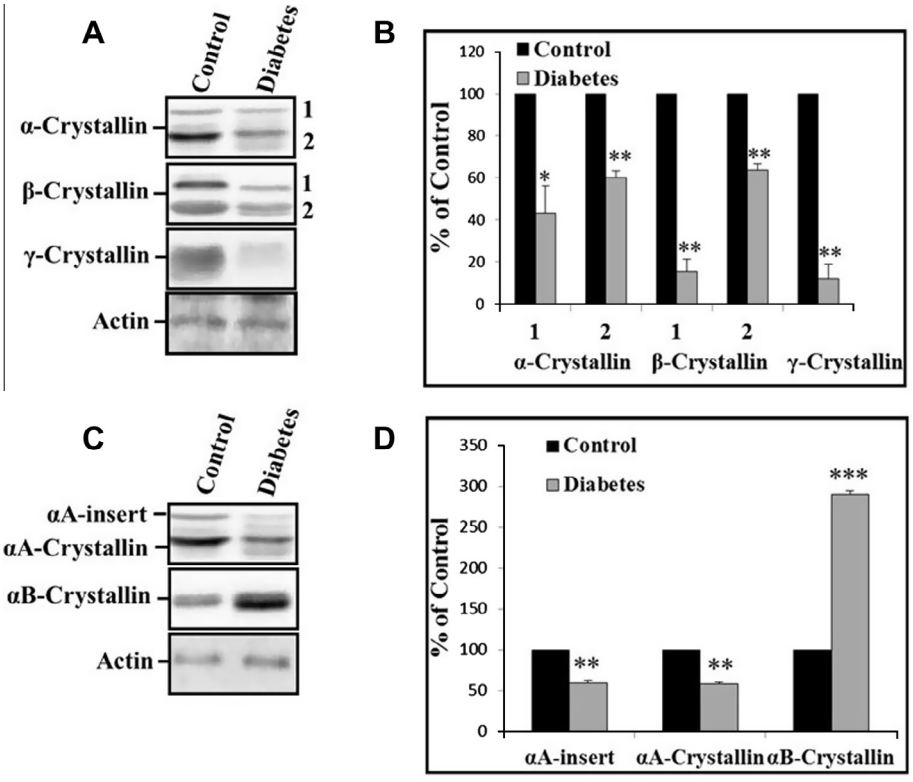

Fig. 1. Crystallin levels in lens of control and diabetic rats. Immunodetection of α, β and γ-crystallins (Panel A) and αAC, and αBC (Panel C). Quantification of α-, β- and γ-crystallin (Panel B) and αAC, and αBC (Panel D) immunoblots in lens of control and diabetic rats; levels was normalized for actin levels and is represented as percent of control. Data represent mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control.

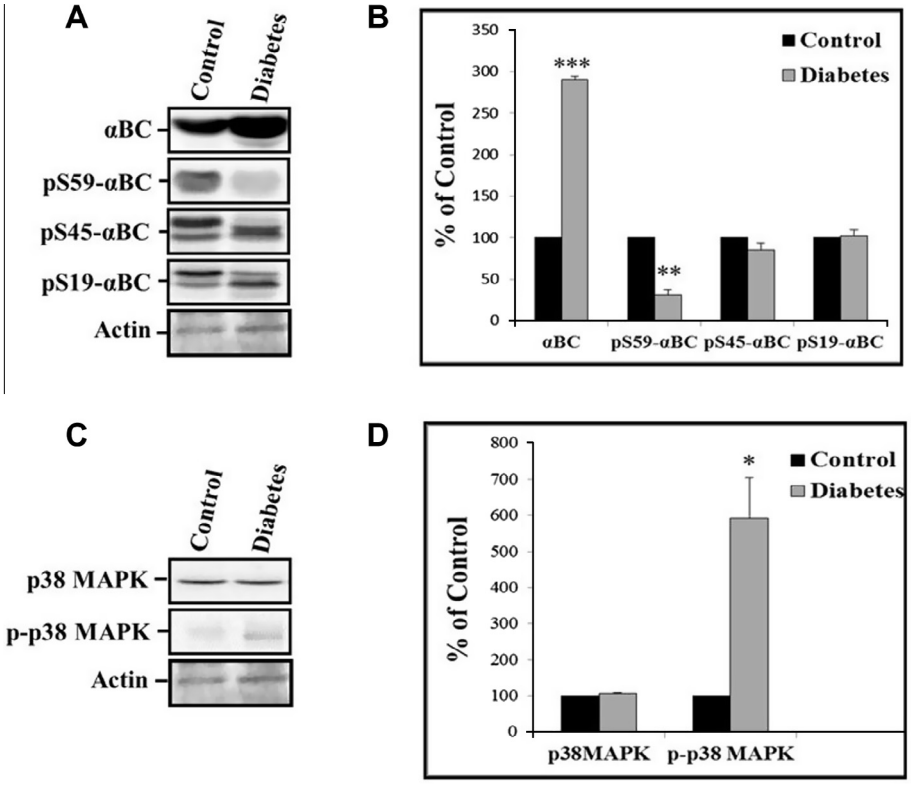

Fig. 2. Phosphorylation of αBC (Panel A) and p38MAPK (Panel C) in lens of control and diabetic rats. Quantification of αBC and pS59, pS45, pS19 of αBC (Panel B) and p38MAPK and p-p38MAPK (Panel D) immunoblots in lens of control and diabetic rats; levels were normalized for actin levels and are represented as percent of control. Data represent mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control.

resulted due to enhanced degradation in diabetic cataract. Furthermore, diabetes activated the p38MAPK signaling cascade by increasing p-p38MAPK levels without increase in total p38MAPK levels supporting the increased phosphorylation at S59 of αBC (Fig. 2C and D).

3.5. Effect of diabetes on solubility of crystallins and phosphorylated αBC

Diabetes could enhance the aggregation of proteins by decreasing the solubility. Therefore, we have investigated the solubility of α, β, γ, αAC, αBC and phosphorylated forms of αBC in lens. Surprisingly, total α-crystallin, β-crystallin and αAC levels were decreased both in detergent-soluble and detergent-insoluble fractions of cataractous lens (Fig. 3). The levels of αBC were increased in detergent-soluble fraction but unaltered in insoluble fraction of diabetic lens (Fig. 3B). However, the levels of γ-crystallins were decreased in detergent-soluble fraction and unaltered in detergent-insoluble fraction (Fig. 3A). Interestingly, increased levels of pS59, pS45 and pS19 of αBC was observed in detergent-insoluble fraction of diabetic lens in comparison with controls (Fig. 3B).

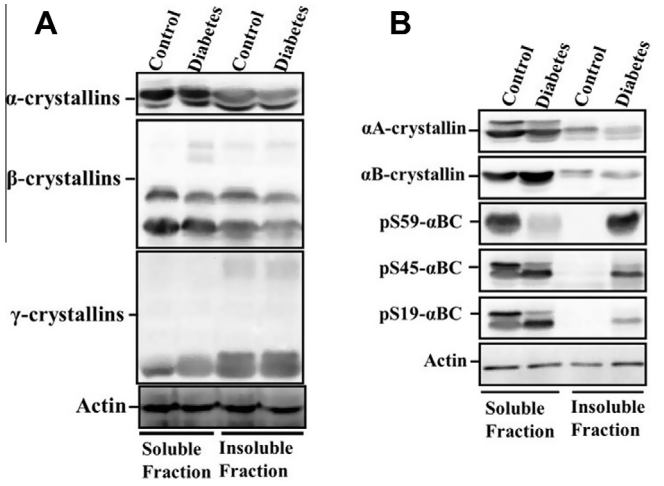

Fig. 3. Solubility of crystallins and phosphoforms of αBC under hyperglycemic conditions. The lens samples were fractionated into detergent-soluble and detergent-insoluble fractions and subjected to immunoblotting. (Panel A) Immunodetection of α-, β- and γ-crystallin in detergent-soluble and detergent-insoluble fractions. (Panel B) Immunodetection of αAC, αBC, αBC-pS59, αBC-pS45, αBC-pS19 in detergent-soluble and detergent-insoluble fraction.

3.6. Interaction of αAC with GRIFIN & αBC

GRIFIN (galectin related inter fiber protein), a galectin superfamily member, shows exclusive levels in lens and is located at the interface between adjacent fiber cells [26]. Previous studies reported that GRIFIN strongly interacts with αAC for maintaining the transparency of lens [27]. Hence, we investigated the GRIFIN levels by immunoblotting. Surprisingly, GRIFIN levels were significantly decreased in diabetic animals in comparison with age matched controls (Fig. 4A and B). Further, the interaction of αAC with GRIFIN was strongly reduced (Fig. 4C). In addition, we also investigated the interactions between αAC and αBC and found that the interaction between αAC and αBC was not affected by prolonged diabetes in lens (Fig. 4C).

In this study, we reported the status of three major crystallins in STZ-induced diabetic cataractous lens. The major findings of the study are: (i) decreased levels of α-, β-, γ-crystallins in diabetic rat lens, (ii) amongst α-crystallins decreased levels of αAC and increased levels of αBC, (iii) presence of all phosphorylated forms of

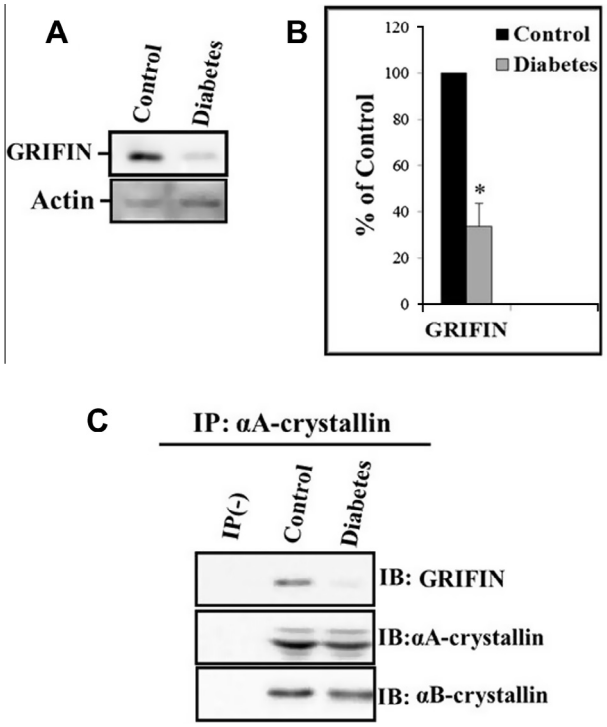

Fig. 4. Interaction of GRIFIN with αAC in lens under hyperglycemic conditions. (Panel A) Immunodetection of GRIFIN in the lens of control and diabetic rats. (Panel B) Quantification of GRIFIN immunoblot in lens of control and diabetic rats; levels were normalized for actin levels and are represented as percent of control. Data represent mean ± SEM of three independent experiments. $^{*}p < 0.05$ compared to the control. (Panel C) Interaction of αAC with GRIFIN and αBC was analyzed by co-immunoprecipitation. IB, immunoblotting antibody; IP, immunoprecipitation; IP (−), no antibody (control immunoprecipitation).

αBC in insoluble fraction (iv) enhanced degradation of α-, β-, αA-crystallins, and phosphorylated forms of αBC (v) altered interaction of GRIFIN with αAC.

Despite the availability of simple and cost-effective surgery, cataracts still comprise a significant risk for visual impairment all over the world, particularly in older individuals. This could be further contributed to by increased prevalence of diabetic cataracts due to increase in number of diabetics. Thus, in addition to good glycemic control, other therapeutic approaches are continuously being sought. Therefore, a greater understanding of molecular basis of the disease might assist in newer and effective preventive and therapeutic approaches.

Hence, we investigated the crystallin levels, phosphorylation and solubility of crystallins which will provide some important clues about their implication in diabetic cataract. The decreased levels of crystallins in both soluble and insoluble fraction can be explained by a couple of factors. The altered protein synthesis or increased posttranslational modifications might be responsible for decreased levels of crystallins in diabetic cataractous lens. Some of the previous studies that reported the decreased crystallin synthesis in galactose induced cataract rat model [28] support the first possibility. However, we have observed that there was a significant increase in αBC but not in αAC expression at transcript levels in diabetic lens [17]. Nevertheless, the hyperglycemic condition makes the crystallins highly susceptible to degradation [17,29] and crystallins have been long shown to be targeted by multiple types of posttranslational modifications in the lens. Hence, these findings strongly suggest that decreased crystallins might be associated with multiple types of posttranslational modifications. Furthermore, several past studies reported the increased glycation and accumulation of AGEs in diabetic rat lens [30–32]. The decrease in levels of γ-crystallins is more when compared with α and β-crystallins in diabetic lens. The speedy glycation of γ- crystallins in comparison with α- and β-crystallins might be responsible for the decrease in diabetes. Though, α- and β-crystallins have more lysine residues than γ-crystallin, they have blocked α-amino groups whereas this site is preferentially glycated in γ-crystallin [33]. Despite the fact that there is a decrease in α-crystallins, the levels of αBC are increased and αAC are decreased in diabetes. It is possible that dominant αAC which exists in 3:1 ratio with αBC might be contributing to the decreased levels of total α-crystallin. The decreased levels of αAC could be due to enhanced degradation, evident from the immunoblot of αAC in diabetic rat lens. Several previous studies reported the enhanced truncation and degradation of αAC under hyperglycemic conditions [29]. The elevated levels of αBC might be associated with increased CLA to mediate correct folding of partially unfolded proteins in diabetic cataract. Previously we reported the sHsp expression along with αAC and αBC in retina of diabetic rats and their levels were differed in retina when compared to lens under chronic hyperglycemia [18]. The αAC levels were increased in the soluble and insoluble fractions of retina whereas decreased in lens in diabetic rat. αBC levels were increased in the soluble and insoluble fractions of retina while increased in soluble fraction of lens and remained unchanged in insoluble fraction of lens. This suggests that αAC might have differential effects and mechanisms in these two different complications of diabetes: cataract and retinopathy.

Phosphorylation at S59, S45 and S19 of αBC is either decreased or unaltered in diabetic rats. The physiological significance of phosphorylation of αBC is unclear. Previous studies reported that intermediate filament vimentin was co-immunoprecipitated with αBC in soluble extracts of bovine lens. Furthermore, in vitro assembly of glial fibrillary acidic protein (GFAP), vimentin was inhibited by αBC; but, the inhibition was independent of phosphorylation [34]. The solubility of phosphorylated forms (pS59, pS45 and pS19) of αBC was compromised under hyperglycemic conditions. Previous studies demonstrated that various types of modifications including phosphorylation [35] are believed to contribute to the aggregation and cross-linking. Taken together, this data suggests that crystallins with increased posttranslational modifications might result in their compromised solubility leading to cataract [32]. Furthermore, modified crystallins become insoluble by aggregation and cross-linking in human lenses during cataractogenesis and aging. The S59 of αBC is phosphorylated by p38MAPK/MAPK activated protein-2 signaling cascade. Here, we show that hyperglycemia increases the p38MAPK phosphorylation in diabetic lens. This finding is in agreement with previous reports of diabetes induced activation of MAPK in lens [36]. Previous studies reported that GRIFIN strongly interacts with αAC for maintaining the transparency of lens [27]. There is not only a decrease in αAC and GRIFFIN but also decreased interaction between these two proteins in diabetic cataractous lens. Decreased levels and interaction of GRIFIN with αAC might be disturbing the efficient packing of the highly concentrated crystallins in the lens.

In summary, we report that chronic hyperglycemia decreased the levels of all crystallins and reduced the solubility of phosphorylated αBC. The altered crystallin synthesis or enhanced degradation, modifications of α-crystallins might affect the chaperone activity and thereby increasing the aggregation and insolubilization of other crystallins under chronic hyperglycemic conditions ultimately resulting in the development of cataract.

Acknowledgments

This work was supported by grants from Department of Science and Technology, Government of India to GBR. VSR was supported by a research fellowship from the University Grants Commission, Government of India. The authors thank Ms. Sneha Jakotia for the help in editing the manuscript.

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