Impact of diabetes on alpha-crystallins and other heat shock proteins in the eye

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Abstract Diabetes and its related complications represent a major growing health concern and economic burden worldwide. Ocular manifestations of diabetes include cataractogenesis and retinopathy, the latter being the leading cause of blindness in the working-age population. Despite numerous studies and recent progress, the exact pathophysiology of the disease remains to be fully elucidated and development of new and improved therapeutic strategies for this chronic condition are greatly needed. Heat shock proteins (Hsps) are highly conserved families of proteins, which are generally regarded as protective molecules that play a wide variety of roles and can be expressed in response to different types of cellular stresses. In recent years, numerous studies have reported their implication in various ocular diseases including diabetic retinopathy. The present review focuses on the potential implication of Hsps in ocular diabetic complications and discusses their specific mechanisms of regulation with respect to their expression, functions and alteration during diabetes. The review will conclude by examining the potential of Hsps as therapeutic agents or targets for the treatment of diabetic retinopathy.

Keywords Hsps · Alpha-crystallins · Diabetes · Ocular complications

Introduction

Since the original report of a heat-induced characteristic pattern of puffing in the chromosomes of *Drosophila busckii*, a

large number of heat shock proteins have been identified. Heat shock proteins (Hsp), also called stress proteins, are a class of proteins whose expression becomes elevated when exposed to a variety of cellular stresses. This dramatic upregulation, or heat shock response, has now been reported in numerous pathological states such as multiple sclerosis, stroke and diabetes. Additionally, their effects have been demonstrated in multiple organ systems, ranging from heart to brain and retina. Heat shock proteins have primarily been described as intramolecular chaperones, folding nonnative proteins to prevent their irreversible aggregation; however, they are also implicated in the regulation of other cellular aspects such as inflammation, metabolism and cell survival. Recent studies have explored and demonstrated the therapeutic potential of specific Hsps. This review will focus on the expression, regulation and function of Hsps in the ocular tissue, how they are affected by diabetes and their therapeutic potential as a treatment of diabetes-related ocular complications.

Expression of heat shock proteins in the eye

Heat shock proteins in the retina

Hsps are some of the most highly evolutionary conserved proteins in living organisms and the heat shock response exists in every organism in which it has been sought, ranging from archaeobacteria to higher eukaryotes including humans. Whereas most features of the heat shock response are similar, there are some variations from species to species such as the optimum temperature at which the response is induced [1]. While they are known for their induction in response to cellular stress, heat shock proteins such as Hsp90 are also expressed under normal conditions and can then represent up

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to 5% of total cellular proteins in eukaryotic cells. In non-pathological conditions, Hsps play a vital role in normal cell function and may possibly form an extension of the cytoarchitecture, assisting cellular traffic of other proteins [2].

Mammalian Hsps are comprised of five major families, which are classified according to their molecular weight: Hsp100, Hsp90, Hsp70, Hsp60 and the small Hsps, which includes Hsp27 (HspB1) and the alpha-crystallins [3]. Although well known for their chaperone activity under stress conditions, Hsps are also involved in many critical cellular mechanisms and functions under normal conditions including development. A constitutive expression of Hsp70 and Hsp90 has been observed throughout development in rat retina [4]. Developmental expression of Hsp27, peaking from postnatal day 6 to postnatal day 12, has been observed in a subset of retinal ganglion cells. This expression correlates with the arrival of late-projecting retinal ganglion cell axons to the superior colliculus and the onset of spontaneous retinotectal activity [3, 5]. In the retinas of 1-day-old piglets, expression of Hsp27 has also been detected, mostly in ganglion cell bodies and some astrocyte processes [6]. These data suggest that Hsps could be involved in the mechanisms responsible for the selection and specific survival of properly connected ganglion cells during the development of the primary visual pathway.

Despite the proteins' nomenclature, it has been shown that their expression is elevated in response to various stresses other than thermal stress. Conditions that trigger their expression are as diverse as oxidative stress, heavy metals, amino acid manipulations or nutrient deprivation [7], UV and gamma irradiation [8], cytostatic and anti-inflammatory drugs [8–11], bacterial and viral infections, and malignant transformation [12]. In line with this notion, the upregulation of heat shock proteins is also very present in the pathologies of many diseases including neurodegenerative and ocular disorders. Hsp27 and Hsp60 have been observed in human eyes from patients diagnosed with either primary open angle glaucoma or normal pressure glaucoma. The specific immunoreactivity for both Hsps was significantly increased in patients with glaucoma when compared to age-matched normal donors, especially in the nerve fiber/ganglion cells and the photoreceptor layers [13]. Hsps are particularly responsive to glaucomatous pathophysiology as illustrated by the increased expression of Hsp90, -70, -60 and -27 in experimentally induced glaucoma in primates [14]. Similar to glaucoma, intraocular pressure (IOP)-induced ischemia affects the function and survival of retinal ganglion cells, while also resulting in an absolute increase in Hsp27 and Hsp72 in ganglion cells during the 2 weeks following the insult [15]. These different studies demonstrate the prevalence of the induction of Hsps in retinal cells exposed to stress.

Crystallins in the retina

Alpha-crystallins belong to the small Hsp family and while they were once only associated with the lens, it has become clear that their expression and role extends to other tissues including ocular ones such as the retina. Like other heat shock proteins, crystallin expression may be instrumental in retinal development. A subpopulation of Müller cells, called peripapillary glial cells (PPGCs), which are located adjacent to the optic nerve head (ONH), expresses alphaB-crystallin during retinal development [16]. AlphaB-crystallin, unlike alphaA-crystallin, is also specifically expressed in the retinal pigment epithelium at specific stages during rat retinal development [17]. Quantitative RT-PCR analysis demonstrated that almost 20 different crystallin genes are expressed in adult mouse retinas. Among those, alphaA- and alphaB-crystallin are expressed at low levels in the ganglion cell layer nuclei, inner and outer nuclear layers and inner segments of photoreceptors in the absence of stress [9, 18–20]. It has been proposed that their expression in retinal photoreceptors contributes to the vectorial transport of rhodopsin from the Golgi apparatus to the rod outer segment [21]. Interestingly, a recent study reported the expression of alphaB-crystallin in retinal pigment epithelium (RPE), pericytes, endothelial cells and astrocytes [22–24], while another study showed that alphaB-crystallin could be secreted by retinal pigment epithelium [25]. The variability of cellular localization in nonpathological conditions might reflect species or strain differences or could be due to environmental conditions.

As previously stated for other Hsps, the expressions of alpha-crystallins are significantly increased in thermal shock and a variety of other stress conditions [26]. Recent studies have shown that levels of alpha-crystallins are considerably elevated in models of light toxicity and retinal tearing and suggested they play a crucial role in the protection of retinal tissue [27, 28]. Alpha-crystallin may also play a significant role in the pathophysiology of neurodegenerative disorders. In a comparative proteomic study examining mouse models of Parkinson's disease, Huntington's disease, the prion disease scrapie and a model of impaired synaptic transmission, alphaB-crystallin was the only protein found to be altered in the brain of the animals of all four neurodegenerative diseases [29]. Interestingly, the levels of expression of either or both alphaA- and alphaB-crystallins have been demonstrated to be increased in a variety of animal models of retinal neurodegeneration including Age-Related Macular Degeneration (AMD), hypoxia, *Staphylococcus aureus*-induced endophthalmitis, experimental autoimmune uveitis (EAU) and diabetic retinopathy [30, 31].

Crystallins and Hsps in ocular tissues

Crystallins were first identified in the ocular lens, where they represent up to 90% of the soluble proteins. The transparency of the lens depends on the high concentration and defined intermolecular interactions of crystallins [32] as demonstrated by the early opacification of the lens observed in alpha-crystallin knockout animals [33]. However, maintaining transparency is not their only function in the lens as there is also a reduction in the survival of lens epithelial cells (LECs) in alphaA-crystallin knockout mice [9]. Other heat shock proteins also help to preserve the transparency of the lens by participating in the folding and maintenance of native proteins. Some of these proteins such as Hsc70, Hsp70 and Hsp40 are detected mainly in the epithelium and superficial cortical fiber cells of the adult human lens. On the other hand, the small heat shock proteins, Hsp27 and alphaB-crystallin, are found in all regions of both fetal and adult human lenses. Aside from their chaperone function, heat shock proteins may have a role in early lens development as indicated by the detection of all heat shock proteins in the fetal human lens [34]. In a study that focused on the localization of Hsc70, Hsp70 and Hsp40 in the lens of embryonic and adult chickens, it was determined that all three were present in the epithelium, cortex and nuclear areas [35].

Proteins of all three nontaxon specific subfamilies of crystallins, alpha-, beta- and gamma-crystallins are expressed in mice and human corneal cells. Crystallin expression in murine corneas starts at an early stage of embryonic development and might last for the entire life of the organism. The level of alphaA-crystallin in corneal epithelium is comparable to that in a developing lens. In the cornea, crystallin expression is responsive to various stressors such as LPS and H₂O₂. It is still unclear whether or not crystallins play an important role in corneal transparency; however, three novel mutations of the alphaA-crystallin gene (CRYAA) at the R116 position have been found to be associated with congenital cataract–microcornea syndrome (CCMC) [36, 37]. In a study examining Hsp expression in 32 human corneas, it was concluded that all Hsps examined, Hsp27, -60, -70 and -90, are expressed in human corneal endothelial cells [38]. The remainder of the review will focus on the expression, regulation and role of Hsps in the ocular manifestations of diabetes mellitus.

Heat shock proteins and diabetes

Expression of heat shock proteins during diabetes in the retina

Diabetic retinopathy, a microvascular complication of diabetes, remains the leading cause of preventable blindness in

the working-age population. It is identified in a third of the people diagnosed with diabetes and is also associated with an increased risk of life-threatening systemic vascular complications including stroke, coronary heart disease and heart failure [39]. Recent studies demonstrated that Hsps and crystallins might play important roles in the pathophysiological mechanism of this complication. The upregulation of crystallin in the retina, especially alphaA- and alphaB-crystallin, has become a hallmark of diabetic retinopathy. This upregulation has been observed in multiple models of both type 1 and type 2 diabetes including streptozotocin- and alloxan-induced diabetes, spontaneously diabetic OLETF and high fat diet-induced diabetic rats [40–42]. Similar to crystallins, several Hsps are also significantly upregulated in the retina during diabetes. In a comparative study of the proteome map of normal and streptozotocin-induced diabetic rat retinas, Hsp70 subtype 1A (Hsp70.1A) along with Hsp70 subtype 8 (Hsp70.8) were two of few uniquely upregulated proteins in the diabetic retina [43]. Despite their established upregulation in various stress conditions, levels of expression of Hsp90 were unchanged while levels of Hsp25 were reduced in the retina of streptozotocin-induced diabetic mice [44]. However, in another study that examined the immunoreactivity of Hsp90 in the retina of OLETF diabetic rats, the levels of Hsp90 were slightly increased at 24 weeks of age [40]. This disparity may illustrate differential roles of Hsp90 in type 1 diabetes versus type 2 diabetes, different roles at various stages of diabetes or interindividual variability in the response to diabetes. The latter point has also been suggested by the analysis of the potential of anti-Hsp27 IgG antibody as a biomarker of microvascular complications of diabetes. Levels of anti-Hsp27 antibody were analyzed in 363 type 1 diabetics and 168 controls leading to the conclusion that they are not a marker of vascular complications in type 1 diabetes [45].

Expression of heat shock proteins during diabetes in other ocular tissues

As previously mentioned and as suggested by their name, crystallins have a predominant role in the lens, especially in the maintenance of its transparency. Unlike what was described in the retina, their expression remains relatively unchanged in the lens of streptozotocin-induced rats compared to that of control rats [31]. It has been long known that diabetic patients are prone to developing cataracts, usually a result of lens protein denaturation. Several publications demonstrated that perturbations of the function of alpha-crystallin proteins are involved in this phenomenon. One such perturbation is nonenzymatic glycation of human lens crystallins; the amount of which was two-fold higher in the diabetic lenses than that in the normal lenses [46]. As further

support of these findings, a study by Nakayama et al. reported a 12-fold increase of advanced glycation end-products (AGEs) in the lenses of streptozotocin-induced diabetic rats at 20 weeks compared to control [47]. It has been shown that aspirin and other nonsteroidal antiinflammatories such as paracetamol and ibuprofen are able to prevent or delay the onset of cataracts during diabetes [48]. Nonsteroidal antiinflammatories can lower fasting glucose levels of diabetics and nondiabetics and diminish glycosuria in diabetic patients and alloxan diabetic rats, which could, in turn, decrease glycation of crystallins and, subsequently, related cataract onset [49, 50]. Furthermore, mutation to alpha-crystallin mimicking some specific posttranslational modifications can lead to cataractogenesis. A R116C substitution in human alphaA-crystallin results in the structural modification of alphaA-crystallin and cataracts possibly by increasing the area of solvent-accessible hydrophobic surfaces normally involved in important subunit interfaces [51]. This increase enhances the probability of protein aggregation in disorders involving protein misfolding [52].

The healing of corneal epithelial wounds is a key component in the maintenance of corneal transparency. Insult to these cells results in the migration of the remaining undamaged corneal epithelial cells to the damaged area. It has been shown that induction of Hsp70 expression may contribute to the promotion of corneal epithelial cell migration and, therefore, corneal wound healing [53]. However, it has been shown that the induction of Hsp70 after injury is delayed in diabetic mice compared to that of nondiabetic mice, resulting in a delay in clinical healing [54]. This may be a result of the impairment of the EGFR–phosphatidylinositol 3-kinase–AKT pathway due to high glucose levels [55].

While not directly related to the alpha-, beta- and gamma-crystallin superfamily, corneal crystallins play a similar role in the cornea. Corneal crystallins, which are highly concentrated in the cornea, have several functions such as maintaining transparency and contributing to the corneal antioxidant systems through a variety of mechanisms [56]. The activation of one corneal crystallin, called Transketolase (TKT), by benfotiamine, a lipid soluble thiamine derivative, may prevent hyperglycemia-induced vascular damage and diabetic retinopathy. It does so by inhibiting three major pathways simultaneously: the hexosamine pathway, the advanced glycation end-product (AGE) formation pathway and the diacylglycerol (DAG)–protein kinase C (PKC) pathway [57].

Regulation of heat shock proteins during diabetes

Regulation of expression

A number of studies have clearly demonstrated that heat shock proteins and, specifically, alpha-crystallins are mainly

regulated at the transcription level during development. In the lens, the regulation of expression of the mouse alphaA- and alphaB-crystallin gene is attained by the control of transcriptional regulatory elements, which reside in their 5' flanking region [58]. A combination of lens lineage-specifying factors such as Pax6 factors, ubiquitously expressed factors including AP-1 family of transcription factors and lens specific factors such as the maf family participate in the regulation of mouse lenticular alphaA-crystallin gene expression [59]. Similarly, ocular heat shock proteins are regulated at the transcriptional level but mostly involve another set of transcription factors called heat shock factors (Hsf). Under stress conditions, Hsfs are relocated to the nuclei, where they form a granulelike structure and bind to heat shock elements (Hses), which are then positioned in the promoter region of the targeted gene, promoting the expression of various Hsps [60, 61]. Interestingly, analysis of the expression of alpha-crystallin transcripts and proteins during diabetes showed that diabetes leads to an increased level of proteins without a correlated increase in their transcript expression [42]. Unlike other retinal pathologies and stages of development, the upregulation of alpha-crystallin in the retina during diabetes is controlled by posttranscriptional regulatory mechanisms. Additional studies are necessary to demonstrate if this upregulation is related to an increased stability of the proteins; however, our recent work showed that a significant portion of alpha-crystallins accumulate in the nonsoluble protein fraction during diabetes, which could lead to decreased degradation [44]. Inversely, the decreased level of expression of Hsp25 could be due to a decreased stability of the proteins or a reduction of transcription and translation rates. Of note, a recent study in rat hippocampal neurons showed that as opposed to alphaB-crystallins, Hsp25 was regulated at the transcription level following stress exposure [62]. This suggests different regulatory mechanisms for Hsps and could explain how diabetes affects various Hsps differently.

Regulation of function

The primary function demonstrated for Hsps is to act as chaperones to protect and help refold damaged proteins following different cellular stresses. We previously mentioned that diabetes differentially affects retinal Hsps at different stages. In addition to changes in their level of expression, the function of Hsps can be modulated through multiple posttranslational modifications. Alpha-crystallins and other Hsps are targeted by a number of posttranslational modifications in the lens and in different retinal diseases including diabetes. In the retina of experimental autoimmune uveitis-induced mice, several proteins including alphaA-crystallin and mitochondrial Hsp70 were targeted by posttranslational modifications [63]. Chemical posttranslational modification of alpha-crystallins and Hsp27 by a

metabolic product, methylglyoxal (MG), has been shown to enhance their chaperone function [64]. In the retinal epithelial cells, it has been concluded that S-glutathionylation of Hsc70 enhances its chaperone function, allowing it to prevent protein aggregation in ATP-free conditions [65]. A wide range of posttranslational modifications targets alpha-crystallins. Preferential glycation has been shown to affect alpha-crystallins in diabetic rat and human lens [66, 67], whereas acetylation has been found to regulate chaperone function of Hsp90 [68]. The increased glycation correlates with an increase in the size of the aggregates formed by alpha-crystallins [67], leading to decreased chaperone activity of the protein [69, 70]. It has also been shown that increased expression of alpha-crystallins in spontaneously diabetic OLETF rats is associated with increased phosphorylation on serine residues 45 and 59 [40]. Interestingly, the phosphorylation on serine residues 45 and 59 of alpha-crystallins negatively regulates their chaperone function [71, 72], which suggests a potential impairment of their chaperone function during diabetes. These results clearly show the importance of posttranslational modifications and how their effect on the functions and regulation of Hsps varies with their nature and specificity.

Overexpression of Hsps and alpha-crystallins has also been demonstrated to be responsible for negatively regulating caspase-dependent apoptosis through a number of mechanisms. First, alphaA-crystallin and Hsp25 were shown to positively regulate the Akt pathway while AlphaB-crystallin was shown to negatively regulate the Raf/Mek/Erk pathway, both of which lead to increased cell survival in lens epithelial cells in culture exposed to UVA light [73–75]. AlphaA- and alphaB-crystallins are known as distinct antiapoptotic regulators. It has been shown that alphaB-crystallin inhibits TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis through the suppression of caspase-3 activation [76]. Another way by which alpha-crystallins prevent apoptosis is by binding to members of the proapoptotic Bcl-2 family, Bax and Bcl-Xs, in order to prevent their translocation as shown in a model of staurosporine-induced apoptosis [77]. It has been shown that expression of Bax and Bcl-Xs are significantly elevated in diabetic retinas while the expression of two antiapoptotic proteins, Bcl-2 and Bcl-Xl, are not changed. Furthermore, despite the elevated levels of alphaA- and alphaB-crystallins, Bax and Bcl-Xs, interactions between these pro- and antiapoptotic proteins were dramatically reduced in the diabetic retina [44]. Whether these changes are due to or responsible for the loss of chaperone function of the alpha-crystallins during diabetes remains to be fully characterized; however, it clearly suggests that their cytoprotective role is impaired during diabetes.

The eye is an immune-privileged site where inflammation is inhibited to prevent damage to the delicate ocular

tissues and preserve vision. However, under specific pathological conditions such as endophthalmitis, uveitis or diabetic retinopathy, the immune privilege is overcome and inflammation is induced within the eye, leading to retinal degeneration. Inflammation is a clear component of the pathophysiology of diabetes and diabetic retinopathy in which Hsps could play a key role. Studies of the impact of endophthalmitis and uveitis in alpha-crystallin knockout animals strongly suggest that alpha-crystallin overexpression could be a special protective mechanism that preserves retinal cells from death during inflammation [30, 78]. However, detection of antibodies against alphaA- and/or alphaB-crystallins in serum from uveitis or multiple sclerosis patients as well as in mice with experimental autoimmune encephalomyelitis [24, 79] could also suggest that alpha-crystallin upregulation in these diseases becomes maladaptive and exacerbate the inflammatory response leading to neurodegeneration. However, the autoimmune attack on alphaB-crystallin is not the direct cause of tissue damage. Rather, it worsens the severity of damage by simultaneously eliminating its action as a restraining element for inflammation and its ability to inhibit programmed cell death [24].

Hsps play critical roles at multiple levels of the pathophysiology of diabetes and its complications. These studies demonstrate that the detailed characterization of their regulation including the posttranslational modifications affecting them is crucial in order to develop novel therapeutic strategies capitalizing on their protective potential.

Potential therapeutic roles

Given the important roles that alpha-crystallins and heat shock proteins play in normal and pathological conditions, their potential as therapeutic agents or targets has recently gained a lot of attention. Despite the previously described regulatory mechanisms and their disruptive effect, two recent studies demonstrated that systemic administration of alphaB-crystallin could rescue cells in models of stroke and retinal ischemia [80, 81]. Administration of alphaB-crystallin 12 h after an experimentally induced stroke was shown to result in both reduced stroke volume and inflammatory cytokines associated with stroke pathology [16, 80]. With a similar approach, Pangratz-Fuehrer et al. demonstrated that alphaB-crystallin administration further enhanced the protective adaptive response following anterior ischemic optic neuropathy by decreasing microglial activation and promoting optic nerve oligodendrocyte survival [81]. Although the mechanisms of action of those treatments still need to be fully elucidated, these findings clearly demonstrate the potential of alpha-crystallins as therapeutic interventions. In the context of chronic disease states such as diabetes, in which the protective functions of alpha-

crystallins seem to be impaired by parallel mechanisms, potential therapies will most likely require the use of strategies involving specifically engineered crystallins [82] or small peptides derived from crystallins, also called “mini-crystallins” [83]. Strategies using other Hsps could also be successful as shown by the reduction of the inflammatory process associated with atherosclerosis that resulted from nasal administration of Hsp65 [84]. A more general approach widely targeting Hsps such as systemic administration of geranylgeranylacetone (GGA), a heat shock protein inducer, could be an interesting option as it has been shown to protect retinal ganglion cells from glaucomatous damage in a rat model [85].

Conclusions

Alpha-crystallins and other Hsps play critical and specific roles in a variety of cellular stress conditions known to take place in the pathophysiology of diabetes. Understanding the exact role and regulation of these proteins in the different ocular tissues could lead to the development of novel therapeutic strategies for diabetic retinopathy and potentially other diabetes complications.

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