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A β PP overexpression and proteasome inhibition increase α B-crystallin in cultured human muscle: relevance to inclusion-body myositis.

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Abstract

Amyloid- β precursor protein (A β PP) and its fragment amyloid- β (A β) are increased in s-IBM muscle fibers and appear to play an important role in the pathogenic cascade. α B-crystallin (α BC) was shown immunohistochemically to be accumulated in s-IBM muscle fibers, but the stressor(s) influencing α BC accumulation was not identified. We now demonstrate, using our experimental IBM model based on genetic overexpression of A β PP into normal culture human muscle fibers, that: 1) A β PP overexpression increased α BC 3.7-fold ($p = 0.025$); 2) additional inhibition of proteasome with epoxomicin increased α BC 7-fold ($p = 0.002$); and 3) α BC physically associated with A β PP and A β oligomers. We also show that in biopsied s-IBM muscle fibers, α BC was similarly increased 3-fold ($p = 0.025$) and physically associated with A β PP and A β oligomers. We propose that increased A β PP is a stressor increasing α BC expression in s-IBM muscle fibers. Determining the consequences of α BC association with A β oligomers could have clinical therapeutic relevance.

Keywords

inclusion-body myositis; α B-crystallin; amyloid- β : amyloid- β precursor protein; cultured human muscle fibers

Introduction

s-IBM, the most common muscle disease of patients age 50 and older, is of unknown etiology and pathogenesis, and it lacks definitive treatment [reviewed in 1]. Light-microscopic features of s-IBM muscle biopsies include vacuolated muscle fibers, accumulation of intra-muscle-fiber multiprotein aggregates, and various degrees of lymphocytic inflammation [2]. Two hypotheses regarding the key pathogenic mechanisms involved in s-IBM are: a) an amyloid- β -related myodegenerative process, and b) an immune dysregulation [reviewed in 2,3]. Intriguingly, the s-IBM muscle-fiber phenotype has similarity to the Alzheimer-disease brain, such as accumulations of multiprotein aggregates containing proteins in congophilic alternate conformation, including amyloid- β (A β) [2]. Reduction of 26S proteasome activity and features of aggresomes were recently demonstrated within s-IBM muscle fibers, and these were modeled in cultured human muscle by experimental overexpression of A β PP [4]. Abnormal increase of A β PP/A β appears to be an early upstream step in the IBM pathogenesis because a) A β PP/A β accumulation appears to precede other detected abnormalities in s-IBM muscle fibers [2], and b) several aspects of the IBM phenotype were produced in cultured normal human muscle fibers (CHMFs) after experimental long-term overexpression of A β PP [5-7]. Long

overexpression of A β PP in muscle of transgenic mice also induced some aspects of the IBM phenotype [8-10]. Moreover, increased oligomerization of A β was associated with increased degeneration of CHMFs [11]. In other cells, A β oligomers are highly toxic, producing more cellular damage than fully fibrillar A β [12-16]. We have postulated that in s-IBM muscle fibers, A β toxicity may not be related to A β in the insoluble aggregates, but rather to a toxicity of its soluble oligomers and protofibrils [2].

α BC, a stress-responsive small heat-shock protein (sHSP), was shown immunohistochemically to be abnormally accumulated in muscle fibers of s-IBM and other myopathies [17]. Of particular interest was the report that in s-IBM, but not in other myopathies, α BC was accumulated not only in the structurally abnormal (vacuolated or otherwise obviously damaged) muscle fibers, but also in many fibers, termed “X-fibers”, which did not display “significant” morphologic abnormality [17], at that or an adjacent level of the muscle fiber (but did have internal nuclei or subtle tinctorial changes in modified trichrome staining in the published photos). It was proposed that increased α BC expression precedes other abnormalities in s-IBM muscle fibers [17]. The stressor(s) inducing α BC in s-IBM fibers was not identified, but it was suggested that α BC might be accumulated due to yet unidentified virus, and as such might play a protective role [17], or it might, either by itself or bound to another protein, induce an inflammatory response [17,18].

Since increased expression of α BC can occur under various stressful conditions [reviewed in 19,20], we hypothesize that in morphologically “undamaged” s-IBM muscle fibers, α BC is induced as a secondary reaction in response to the early increase in them of “morphologically invisible” soluble A β -oligomers (not in aggregates). To test this hypothesis, we studied α BC in cultured human muscle fibers shortly after A β PP-overexpression in them, before typical structural abnormalities developed. We also evaluated, both in CHMFs and in s-IBM muscle fibers, whether α BC physically associates with A β PP and A β oligomers. In this manuscript, we demonstrate the following: 1. By immunoblots, α BC is increased in a) CHMFs due to A β PP-overexpression and proteasome inhibition, and b) biopsied s-IBM muscle fibers. 2. In both, the CHMFs and s-IBM muscle fibers, α BC physically associates with A β PP and A β oligomers.

Material and Methods

Cultured human muscle fibers (CHMFs)

Cultures were established from satellite cells of archived portions of diagnostic muscle biopsies from patients who, after all tests were performed, were considered free of muscle disease [21].

In six culture sets, each established from a different biopsy, a 3 Kb 751 A β PP-cDNA, in either sense or anti-sense orientation, was transferred into three-week-old cultured muscle fibers, using a replication-deficient adenovirus (RDAdV) vector, at 0.3×10^8 pfu/ml culture medium, as detailed [4-7]. (In this culture model, an increase of A β PP and A β is visible by immunoblots and Elisa within 24-48 hours after A β PP gene-transfer, and morphologic aspects of IBM appear approximately 14-21 days after the transfer.) Three days after transfer, experimental and control (non-A β PP-overexpressing) cultures were treated with 1 μ M epoxomicin (Biomol Research Laboratories, Plymouth Meeting, PA), an irreversible proteasome inhibitor [22]. 24 hours thereafter, the cultures were processed for light-microscopic immunocytochemistry, immunoblotting, and combined immunoprecipitation/immunoblotting, as described [4-7,11, 23,24].

Light-microscopic immunocytochemistry

This utilized two antibodies against α BC: mouse monoclonal clone 1b6.1-3G4 (Stressgen Bioreagents, Victoria, BC), diluted 1:250 [17]; or a rabbit polyclonal antibody (Stressgen), diluted 1:250. Rabbit polyclonal antibody against γ -tubulin (Santa Cruz, CA), diluted 1:150, was used to identify aggresomes [4].

Immunoblots

These were performed as described [5,7,11,23,24]. In brief, 10 μ g of protein were loaded onto 12% NuPAGE gels (Invitrogen, Grand Island, NY), electrophoresed in MES-SDS NuPAGE buffer, transferred to nitrocellulose membranes, and immuno-probed with mouse monoclonal antibodies against α BC, diluted 1:1000. The blots were developed using the enhanced chemiluminescence system. Protein loading was evaluated by GAPDH and actin bands, visualized with a goat polyclonal anti-GAPDH antibody (1:400), and a mouse monoclonal anti-actin antibody (1:2000) (both from Santa Cruz, CA). Quantification of immunoreactivity was performed in 6 culture sets by densitometric analysis using the Kodak Gel Logic 440 imaging system (Eastman Kodak Company, Rochester, NY).

Combined Immunoprecipitation-Immunoblotting

To evaluate whether, in A β PP-overexpressing ("A β PP+") CHMFs, α BC physically associates with A β PP and A β oligomers, we performed a 2-stage-combined immunoprecipitation-immunoblot technique, as described [23,24]. In brief, 100 μ g of total muscle protein was immunoprecipitated in precipitation-buffer containing either 5 μ g of 6E10 antibody against A β PP/A β (Signet, Dedham, MA), or 5 μ g of antibody against α BC. The reaction mixture was incubated overnight at 4°C on an orbital shaker. The immunoprecipitated complex, containing IgG antibody, along with its bound target antigen and all proteins bound to that antigen, was pulled down using Protein G Sepharose 4 Fast Flow (Amersham Biosciences Corp. Piscataway, NJ) and incubating for 4 hours at 4°C. That solution was centrifuged for 5 minutes (16,000xg at 4°C) and supernatant removed. The precipitated sepharose immunocomplexes were three times washed with the precipitation buffer by centrifuging 5 minutes (16,000xg at 4°C). To break all bonds between antibody and target-antigen, and between target-antigen and its in vivo binding partners, the pellet was resuspended in 48 μ l of 4X NuPAGE LDS sample buffer and 12 μ l of 10X sample reducing agent (both Invitrogen, Carlsbad, CA), boiled for 10 minutes and then centrifuged for 5 minutes (16,000xg at 4°C). 10 μ l of the supernatant was loaded onto 12% NuPAGE gels (Invitrogen, Grand Island, NY), electrophoresed in MES-SDS NuPAGE buffer, and transferred to nitrocellulose membranes. The electrophoresed protein products of the α BC-immunoprecipitate were immunoprobed with 6E10 anti-A β PP/A β antibody, and the products of the A β PP/A β immunoprecipitate was immunoprobed with anti- α BC antibody, each followed by an appropriate secondary antibody. To confirm specificity of the immunoprecipitation reaction, primary antibodies were omitted from the immunoprobings.

Biopsied muscle fibers

To evaluate whether α BC binds to A β PP/A β in s-IBM muscle biopsies, we performed α BC immunoblots and combined-immunoprecipitation-immunoblots of fresh-frozen diagnostic muscle biopsies obtained with informed consent. Five biopsies were s-IBM and 4 were normal with all our diagnostic studies. All diagnoses were based on our routinely-performed extensive clinical and laboratory studies [1].

Immunoblots

Immunoblots were performed as described above for CHMFs and recently detailed [4,23-25].

Combined immunoprecipitation-immunoblot procedure

To evaluate if in s-IBM muscle fibers α BC physically associates with A β PP and/or A β , a combined immunoprecipitation-immunoblot technique was performed, essentially as above and as described [4,23-25]. In brief, 100 μ g of total muscle protein from s-IBM and control muscle biopsies were individually immunoprecipitated in precipitation buffer containing 5 μ g of anti- α BC antibody. The immunoprecipitates were electrophoresed: a) on 12% NuPAGE gel in MES-SDS NuPAGE buffer to visualize A β oligomers, and b) on 4-12% NuPAGE gel in MOPS-SDS NuPAGE buffer to visualize A β PP. All other steps were as described above for CHMFs.

Statistical Analysis

These analyses were performed in all experiments using Student t-test. Significance level was set at $p < 0.05$. Data are reported as means $\pm$ SEM for all groups.

Results

Cultured human muscle fibers

Light-Microscopic Immunocytochemistry—In the cytoplasm of A β PP+ CHMFs, α BC was diffusely and more strongly immunoreactive than in control CHMFs (Fig.1 A,B). In about 80-90% of A β PP+ CHMFs, epoxomicin treatment induced large strongly-immunoreactive α BC aggregates, which colocalized with γ -tubulin, indicating that they were aggresomes [4] (Fig.1 C,D).

Immunoblots—In all culture sets, α BC migrated as a 22 kDa band (Fig. 2A). Compared to untreated control cultures, α BC was increased: a) 3.7-fold ($p = 0.025$) in A β PP+ CHMFs; b) 3.4-fold ($p = 0.001$) in epoxomicin-treated control CHMFs; and c) 7-fold ($p = 0.002$) in epoxomicin-treated A β PP+ CHMFs (Fig.2 A, B). CHMFs overexpressing antisense A β PP did not have increased α BC (Fig. 2 C,D).

Combined immunoprecipitation-immunoblotting—Immunoprecipitation of proteins from A β PP+ CHMFs with 6E10 antibody, which recognizes both free A β and A β PP, followed by immunoprobng with an antibody against α BC, revealed a very strong α BC band of 22 kDa (Fig. 2E). Conversely, immunoprecipitation with an antibody against α BC, followed by immunoprobng with 6E10 antibody, revealed a strong band at 130 kDa corresponding to A β PP, and bands at 8, 12 and 16 kDa corresponding to A β oligomers (dimers, trimers, and tetramers) (Fig.2 F,Fa). Those results indicate that α BC physically associates with A β PP and free A β oligomers. A 4kDa A β monomer was not identified. (The 26 and 50kDa bands of IgG resulting from the immunoprecipitation reactions are non-specific bystanders.)

Biopsied muscle fibers

Immunoblots—As in CHMFs, α BC migrated as a 22kDa band both in control and s-IBM muscle biopsies. As compared to controls, in s-IBM muscle fibers, α BC was increased 3-fold ($p = 0.025$) (Fig.2 G,H).

Combined immunoprecipitation-immunoblotting—Immunoprecipitation of s-IBM muscle fibers with an antibody against α BC, followed by immunoprobng with 6E10 antibody, revealed a strong band at 130 kDa corresponding to A β PP (Fig.2 Ia) and two bands at 8 and 16 kDa corresponding to A β dimers and tetramers (Fig.2 Ib). A β monomer and trimer bands were not visible under these conditions. Control muscle fibers were negative. Those results indicate that in s-IBM muscle, similarly to CHMFs, α BC physically associates with A β PP and A β oligomers.

Discussion

As an sHSP, α BC is known to play a role in the cellular defense against improperly degraded, accumulated and toxic proteins [19,20]. α BC specifically recognizes and stabilizes proteins that have a propensity to aggregate and precipitate [19,20]. In Alzheimer brain, α BC binds to A β PP [26], and α BC mRNA is increased (referenced in [26]). In vitro, α BC prevents A β fibril growth and spontaneous fibril formation, binds A β , and prevents its aggregation [27-29]. However, when applied extracellularly to cultured rat neurons, concomitantly with A β , α BC increases A β cytotoxicity [28], possibly due to α BC's influence in maintaining A β in its soluble oligomeric, highly cytotoxic form [28]. Several studies have demonstrated that A β and other amyloidogenic proteins exert their cytotoxicity when in the form of oligomeric intermediates or "pre-amyloid" protofibrils, and not while in the form of insoluble amyloid fibrils or aggregates [12-16].

In Alzheimer disease, A β oligomers are now considered more harmful than organized amyloid plaques [12-16]. Our studies of cultured human muscle fibers bearing the transthyretin Val122Ile mutation and overexpressing A β PP indicated that increased muscle fiber degeneration is associated with increased A β oligomerization [11]. Of interest is a recent study related to cultured cardiomyocytes which experimentally expressed both α BC bearing the R120G mutation, causing desmin-related myopathy [30], and a toxic amyloidogenic peptide PQ81 [31]. Those cardiomyocytes had pronounced aggresome formation but, interestingly, inhibition of the aggresome formation through additional expression of the wild α BC led to increased accumulation of PQ81 amyloidoligomers and increased cellular toxicity [31]. That study suggests that, while a mutated α BC causes aggregation of a toxic amyloidogenic protein, prevention of aggresome formation by maintaining amyloidogenic protein in the form of oligomers was associated with increased toxicity [31].

Our current study provides a novel demonstration that in human muscle fibers α BC is increased due to: a) A β PP overexpression (A β PP+), b) proteasome inhibition, and c) a combination of both has an additive effect. Moreover, α BC physically associates with A β PP and A β oligomers in human muscle fibers, both in A β PP+ cultures and in s-IBM muscle fibers. Our immunoblot studies also confirmed what was previously reported by immunohistochemistry [17], that α BC is significantly increased in s-IBM muscle fibers. In our culture IBM model, we studied α BC shortly after A β PP overexpression and before morphologic abnormalities were evident. We raise the possibility that the binding of α BC to A β oligomers might retard and diminish their fibrillization and aggregation into visible aggregates, thereby prolonging their existence as toxic oligomers (if α BC binding per se does not detoxify the oligomers). Those cultured muscle fibers mimic the normally-appearing IBM biopsied muscle fibers that have α BC increase without "significant" morphologic abnormalities [17]. Proteasome inhibition, which appears to be an important part of the s-IBM pathogenesis and in A β PP+ CHMFs, results in aggresome formation [4], additionally increased α BC expression and, in A β PP+ CHMFs, caused α BC accumulation in aggresomes, as previously shown in human glioma cells [32]. (In our studies, increased α BC in A β PP+ CHMFs is not due to the adenovirus transfer vector because using the same vector to transfer antisense A β PP cDNA did not result in increased α BC).

In summary, this experimental study 1) identified A β PP and/or oligomeric A β , its toxic proteolytic product, as probable stressors causing increased α BC in s-IBM muscle fibers that morphologically appear without significant damage, and 2) again supports an important upstream role of A β PP/A β in the s-IBM pathogenesis. Moreover, proteasome inhibition, which is present in s-IBM muscle fibers and is experimentally produced by A β PP overexpression in CHMFs [4], might additionally contribute to the α BC increase in s-IBM. Based on our data, and evidence of others in neuronal tissue [28], we propose that in s-IBM fibers α BC binding to A β oligomers with the "intent" to chaperone actually may be detrimental by preventing

A β oligomers from assembling into the putatively non-toxic aggregates. Another possibly detrimental effect is that α BC bound to A β PP/A β might help induce or maintain the inflammatory reaction in s-IBM muscle [18]. Those putatively adverse reactions, if confirmed, could provide novel therapeutic targets. Alternatively, a beneficial α BC chaperoning effect in s-IBM muscle fibers can also be considered if the α BC binding is to a cytotoxic moiety of A β PP/A β . Our studies do not negate the possibility, as was previously suggested [17], that in s-IBM muscle fibers α BC might also be induced by other stressors, for example a yet unidentified virus, which has been proposed to play a role in the s-IBM pathogenesis [reviewed in 1-3] and which could be antecedent to the increased expression of A β PP/A β .

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Fig1.

Single (A,B) and double (C,D) immunofluorescence of cultured human muscle fibers (CHMFs). A-C- α -BC; D- γ -tubulin. In A β PP-overexpressing CHMFs (B) α BC is strongly immunoreactive in contrast to control CHMFs (A), which have very weak α BC-immunoreactivity. Epoxomicin treatment induced large α BC-immunoreactive aggregates in an A β PP-overexpressing CHMF (C), which colocalized with γ -tubulin (D), indicating that they are aggresomes. All $\times$ 1,100.

Cultured human muscle fibers.

* Omission of primary antibodies from immunoblotting.

Fig2.

Cultured human muscle fibers. A-immunoblot of CHMFs lysates, using an antibody against α BC, illustrates increased α BC in epoxomicin-treated, in AβPP-overexpressing (AβPP+), and in combined AβPP+ and epoxomicin-treated cultures, as compared to controls. In the immunoblotting, omission of the primary antibody (*) resulted in no 22kDa band. GAPDH and actin illustrate protein loading.

B-Densitometric analysis of six blots, as in "A", relative to GAPDH. C, D-an immunoblot and a densitometric analysis of control cultures and those to which AβPP cDNA was transferred in antisense orientation -- these show that antisense AβPP cDNA did not increase α BC.

E, F-immunoprecipitation (IP) experiments. E-IP of A β PP+ CHMFs with 6E10 antibody recognizing A β PP/A β , followed by immunoblotting with an antibody against α BC, demonstrates a 22kDa band corresponding to α BC. In the immunoblotting, omission of the primary antibody (*) resulted in no 22kDa band. 26 and 52 kDa bands correspond to immunoglobulin G, a nonspecific bystander in the immunoprecipitation reactions. F-IP of A β PP + CHMFs with an antibody against α BC, followed by immunoblotting with 6E10 antibody recognizing A β PP and A β , shows a strong 130kDa band corresponding to A β PP and three weak 8, 12 and 16 kDa bands corresponding to A β oligomers; Fa-a lower part of "F", exposed 3 times longer to better visualize A β oligomers. 26 and 50 kDa bands correspond to immunoglobulin G and are nonspecific; there were no specific bands when the primary antibody was omitted.

Muscle biopsies. G-representative immunoblot of 2 control (C) and 2 s-IBM muscle homogenates shows increased α BC bands in s-IBM as compared to controls. H-Densitometric analysis of immunoblots as in G, based on 4 control and 5 s-IBM muscle homogenates, demonstrates 3-fold increase of α BC in s-IBM. There were no specific bands when the primary antibody was omitted (*).

I-immunoprecipitation of control (C) and s-IBM muscle homogenates with an antibody against α BC, followed by immunoblotting with 6E10 antibody recognizing A β PP and A β ; this shows in s-IBM a strong 130kDa band corresponding to A β PP (a) and two bands, 8 and 16 kDa, corresponding to A β dimer and tetramer (b). There were no specific bands when the primary antibody was omitted (*). Normal control is negative.