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Intracellular Delivery of Recombinant alpha B-crystallin into Neonatal Rat Cardiomyocytes has a Protective Effect on the Cells

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ABSTRACT

In order to deliver alpha B-crystallin (alpha B-C) into cardiomyocytes and study its cellular protection, the full-length cDNA fragment encoding human alpha B-C was cloned into the bacterial expression vector pGEX-MTS containing the base sequence of membrane-translocating sequence (MTS) which mediates intracellular delivery of peptides and expressed as a fusion protein coupled to glutathione S-transferase (GST).After glutathione affinity chromatography and cleaved from GST by factor Xa, the recombinant MTS- alpha B-C was separated from GST and factor Xa by anion exchange chromatography. Recombinant MTS- alpha B-C was characterized by SDS-PAGE and Western immunoblot analysis. The purified MTS- alpha B-C migrated on SDS-PAGE as a single band to an apparent molecular weight (Mr.23kD) that corresponded to total native alpha B-C plus MTS, and was recognized on Western immunoblot by anti-human alpha B-crystallin antibody. MTS- alpha B-C displayed chaperone-like function in an ATP-containing buffer at 37℃ by disaggregating the denatured and aggregated actin induced by hydrogen peroxide (H_2O_2)treatment. It was observed under fluorescence microscope that FITC-labeled MTS- alpha B-C had gone into neonatal rat cardiomyocytes by MTS mediation after the cells were incubated with it for 6 hours while FITC-labeled alpha B-C and bovine serum albumin had not gone into the cells. Recombinant MTS- alpha B-C is not cytotoxic, and MTS- alpha B-C-treated cells displayed increased H_2O_2-tolerance compared with non-treated cells.

Key words: alpha B-crystallin; Protein engineering; Membrane-translocating sequence; Cardiomyocytes; Cytoprotection

INTRODUCTION

Life processes of cells are regulated with precision by various endogenous proteins. In order to deeply study and manipulate these processes, many methods have been utilized for raising the concentration of specific proteins within cells, such as gene transformation, microinjection and liposome package (Indraccolo et al . 2001; Aoi et al. 2000 ; Iwata et al. 2001).

Heat shock proteins, also termed molecular chaperones, are a superfamily of proteins which can be synthesized or up-regulated within cells under stress conditions, and their basic functions are to help other proteins maintain or restore the correct folding states, participate in translocation of other proteins, and promote degradation of irreversibly denatured proteins (Ghosh et al. 2006; Melkani et al. 2006; Ghosh et al. 2005). Alpha B- crystallin (alpha B-C) is a small heat shock protein with approx. molecular weight of 22kD, abundant in cardiomyocytes comprising about 2% of cellular soluble proteins(Kato et al. 1991). In normal conditions, alpha B-C is distributed throughout cytoplasm as a soluble protein, but during myocardial ischemia or ischemia/reperfusion it assembles into a high molecular weight oligomer and translocates to Z-line and I-band of myocardium, and in the meantime it is obviously up-regulated to maintain the stability of myocardium against ischemia or ischemia/reperfusion injury(Golenhofen et al. 1998; Golenhofen et al. 1999; Ray et al. 2001). In view of the chaperone-like function of alpha B-C in conjunction with its high level of constitutive expression in the myocardium, it was assumed that alpha B-C, translocated to the Z-line area during myocardial ischemia or ischemia reperfusion injury, might assist certain myofibrillar components at this site to maintain their correctly folded state and to prevent them from unfolding and loss of function (Golenhofen et al. 1999). In current opinions, alpha B-C can protect cells during stress conditions against injury although the molecular mechanism of its protection is unclear (Eaton et al. 2000; Nefti et al. 2005). Considering that alpha B-C is an intracellular protein, if we can deliver exogenous alpha B-C into cells, we can not only investigate its functions, but also may make progress in putting it into clinical practice .

Membrane-translocating sequence(MTS), a 12-amino acid residue peptide, originating from the hydrophobic region (h region) of a signal peptide sequence of Grb2 (SH2 domain) has been used as a carrier to deliver various peptides (the cargo) into living cells in a nondestructive manner(Rojas et al. 1998), but we do not know if it can carry alpha B-C, a large oligomer-forming protein, into living cells. In this study, MTS was fused to the N-terminus of human alpha B-crystallin, and the resultant MTS-alpha B-C fusion protein were efficiently imported into cardiomyocytes by MTS mediation. Although there is great basal expression of alpha B-C in cardiomyocytes, the imported alpha B-C showed a significant protective effect on the targeted cells. Our research for the first time demonstrated that recombinant human alpha B-C with MTS could be delivered into living cardiomyocytes and protect the cells against injury induced by exposure to hydrogen peroxide.

MATERIALS AND METHODS

Chemicals and Materials

All materials for cell culture were from Gibco BRL. Sepharose4B, FactorXa and SephadexG-25 were purchased from Amersham Pharmacia Biotech. Cellulose DEAE-52 was from Whatman. Fluorescein isothiocyanate and acrylamide were purchased from Amersco. Bis-acrylamide and Coomassie Blue R-250 and G-250 were purchased from Fluka. Bovine serum albumin was from Serva, Germany. Reduced glutathione was from Japan. IPTG was from BBI. Protein molecule weight marker was from Bio-Rad. SDS and alpha B-crystallin and HSP25 were from Sigma. Anti-human- alpha B-crystallin antibody was from Stressgen. Other chemicals all were of analytical grade. Bacterial expression vector pGEX-MTS was kindly provided by Mr.Yao-Zhong Lin at Vanderbilt University, USA. Bacterial expression vector pRF14G containing full length cDNA of alpha B-C was kindly provided by Dr. Ivor Benjamin at University of Texas Southwestern Medical Center, USA. Wistar or SD neonatal rat is from the Animal Center of Central South University.

Preparation of reduced gutathione-Sepharose 4B

The reduced glutathione-Sepharose 4B was prepared according to Simons PC(Simons et al. 1977) with some modifications. Briefly, 35g of Sepharose 4B was washed on Buchner funnel with 2000 ml of distilled water, then suction-dried. The suction-dried Sepharose was transferred to a 2L round-bottom flask containing 56 ml of sodium hydroxide (1 mol/L), 5.6 ml of epichlorohydrin. The reaction mixture was stirred in a water bath at 60℃ for 2 hours, after which the epoxy-activated gel was transferred to a Buchner funnel and washed with 2000 ml of distilled water and 100 ml of 0.05mol/L Na phosphate (pH 7.2) accordingly, and suction-dried finally. The epoxy gel was again transferred into an 1L round-bottom flask containing 40 ml of reduced glutathione (80 mmol/L) in 0.05 mol/L Na phosphate (after addition of the reduced glutathione to 0.05mol/L Na phosphate, the buffer solution was adjusted to pH 7.2 with 2 mol/L NaOH). The reaction mixture was stirred in a water bath at 37℃ for 20-24 hours. The affinity gel prepared in this way was washed with distilled water and then stored in distilled water containing 0.02% NaN_3 at 4℃ for future use.

Construction of MTS- alpha B-C expression vector

Bacterial expression vector pGEX-MTS containing sequences of both glutathione-S-transferase and membrane-translocating sequence was linearized with EcoRI. The full length sequence of human alpha B-C cDNA from vector pRF14G was also digested with EcoRI and cloned into the linearized bacterial expression vector pGEX-MTS to produce pGEX-MTS- alpha B-C, resulting in the expression of a GST-MTS- alpha B-C fusion protein. The coding region of this expression construct was confirmed by DNA sequencing analysis (Shanghai BoYa Biotech Company Ltd).

Expression and isolation of GST-MTS-alpha B-C fusion protein

The pGEX-MTS-alpha B-C expression plasmid was used to transform competent E. coli DH5a cells(Muchowski et al. 1997; Smith et al. 1988). One liter of L broth that contained 100μg/ml ampicillin was inoculated with 5ml of an overnight culture of the transformed E. coli cells, and cells were grown with vigorous shaking at 37℃ until the culture reached an optical density of 0.7 at A=600nm, at which point protein expression was induced by addition of IPTG to a final concentration of 1mM. Three hours after induction, cells were harvested by sedimentation and resuspended in 30 ml of PBS containing 4 mol/L urea. Cells were stored overnight at -20℃. After thawing, the cells were disrupted by sonication on ice. Insoluble debris was removed by sedimentation at 10000xg for 10 min at 4℃. The supernatant was dialyzed against PBS to remove the urea, and soluble fusion protein present in the supernatant was isolated by adsorption to 20 ml glutathione-Sepharose 4B affinity column at 25℃. After washing with PBS until $O.D_{280}$ of the flowthrough <0.02, bound fusion protein was eluted using PBS containing 10mM reduced glutathione. The fusion protein prepared in this way contained a few contaminating proteins displayed by SDS-PAGE and Coomassie Blue staining. Concentrations of isolated fusion protein were determined by Bradford method.

Purification of recombinant MTS-alpha B-C

GST was cleaved from GST-MTS- alpha B-C using the protease Factor Xa, the recognition sequence of which is encoded in the pGEX-MTS- alpha B-C vector. Recombinant MTS-alpha B-C was purified by anion exchange chromatography in the presence of 8M urea. Briefly, cleaved fusion protein was dialyzed against ion exchange buffer (20 mmol/L TrisHCI, pH8.0, 25 mmol/L NaCl, 1mmol/L EDTA, and 8 mol/L urea). GST, Factor Xa, GST-MTS- alpha B-C and MTS- alpha B-C were all absorbed to a 50 ml column of cellulose-DEAE-52 anion exchange resin in the presence of ion exchange buffer, but recombinant MTS- alpha B-C was eluted in the first peak and other proteins in other peaks when elution was performed with a linear salt gradient ranging from 0~0.3 mol/L NaCl. Preparations of recombinant MTS- alpha B-C were dialyzed against ion exchange buffer that lacked 8mol/L urea to promote reoligomerization and then concentrated by superfiltration on Amenion membrane. Preparations of recombinant MTS- alpha B-C were found to contain <5% contaminating proteins as assessed by SDS-PAGE and Coomassie Blue staining. Concentrations of purified MTS- alpha B-C were determined by Bradford method.

SDS-PAGE and Western immunoblot analyses of MTS- alpha B-C

SDS-PAGE was done according to the method of Laemmli (Laemmli. 1970). Western immunoblot analysis was done according to Pharmacia Biotech instructions.

Disaggregation effect of MTS- alpha B-C on denatured proteins

For detection of chaperone-like function of MTS- alpha B-C, 100μ g of myocardial proteins determined by Bradford method was added to 50μl of 200mM H_2O_2(Goloubinoff et al. 1999).This reaction mixture was then incubated at 37℃ for 30 min. Protein precipitates were harvested by sedimentation at 1000xg for 15 min at 4 ℃. 2μg of MTS- alpha B-C or HSP25 was added to the protein precipitates, and the mixtures were adjusted to a final volume of 30μ l with a buffer (140 mmol/L NaCI,pH7.4, 5 mmol/L Na phosphate containing ATP).After incubation at 37℃ for 4 hours, the precipitates were separated from the supernatants by sedimentation at 10000 x g for 15 min at 4°C. Actins in both the precipitates and supernatants were analyzed by Western immunoblot respectively. The amount of actin in the supernatants was used to evaluate the chaperone-like activity of MTS- alpha B-C.

FITC-labeling of MTS- alpha B-C

Fluorescein isothiocyanate(FITC) was labeled to purified MTS- alpha B-C, alpha B-C and bovine serum albumin (BSA) as described in the literature(Beijing Medical College. 1980). Gel filtration of Sephadex G-25 was used to separate the FITC-proteins from free FITC.

Cardiomyocytic import of MTS- alpha B-C

Confluent neonatal rat cardiomyocytes were incubated with a final concentration of 0.6μ mol/L of the purified FITC-labeled MTS- alpha BC or alpha BC or BSA in DMEM at 37 ℃ for 6 hours; FITC- alpha B-C and FITC–BSA were used as negative controls. The cells were extensively washed, and then put under fluorescence microscope to observe whether the proteins had entered the cardiomyocytes.

Cytotoxicity assay

1, Freshly prepared Trypan Blue in PBS/DMEM was added to the MTS- alpha B-C -treated or untreated cells in culture dishes at room temperature for 5 min. Cells were then observed by a bright-field microscope and Trypan Blue-stained cells were counted as not viable. 2, MTS- alpha B-C-treated or untreated cells were trypsinized and separated by sedimentation at 100rpm at room temperature for 10 min from the culture medium. Cells were disrupted by sonication on ice. Insoluble cellular debris was removed by sedimentation at 10000×g for 10 min at 4°C. The lactate dehydrogenase (LDH) activities of the supernatant and the culture medium then were determined directly by a test kit(produced by Beijing Bo Ding Biotech Co.,Ltd). The ratio of LDH activity in medium to that in cells (LDH release ratio) was used as a parameter to evaluate the cytotoxicity.

Protective effect of MTS- alpha B-C on cardiomyocytes

Freshly prepared H_2O_2 in DMEM (0.5 mmol/L) was added to the MTS- alpha B-C -treated or -untreated cells in culture dishes at 37 ℃ for 3 hours. The death

rate and LDH release ratio were determined as above and used to judge whether the imported MTS- alpha B-C showed a protective role.

Statistics

Results are presented as mean ±sd. In all experiments, *n* equals the number of samples. Differences between groups were assessed by *t*-test, and <0.05 p value was considered significant.

RESULTS

Characterization of MTS- alpha B-C cDNA clone

The cDNA-coding region of human alpha B-C from EcoRI-digested pRF14G was ligated into the plasmid pGEX-MTS to produce pGEX-MTS- alpha B-C. EcoRI-digested parental plasmid pGEX-MTS produced only big DNA fragment, namely pGEX-MTS, but EcoRI-digested recombinant plasmid pGEX-MTS- alpha B-C produced an about 700bp alpha B-C cDNA fragment besides pGEX-MTS vector(Fig.1A and B). Sequencing of both strands encoding pGEX-MTS- alpha B-C by Shanghai Bo Ya Biotech Company Litd confirmed that DNA sequence of GST- MTS- alpha B-C fusion protein in recombinant plasmid pGEX- MTS- alpha B-C was in the same reading frame.

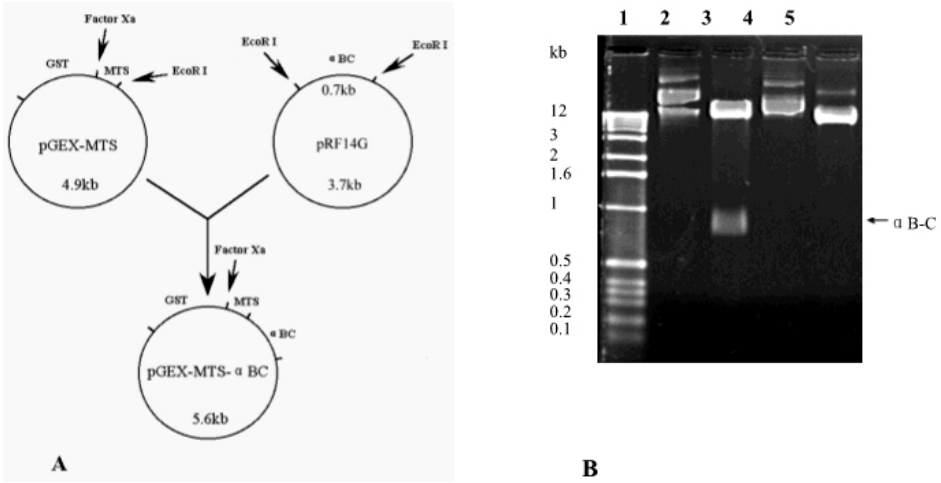
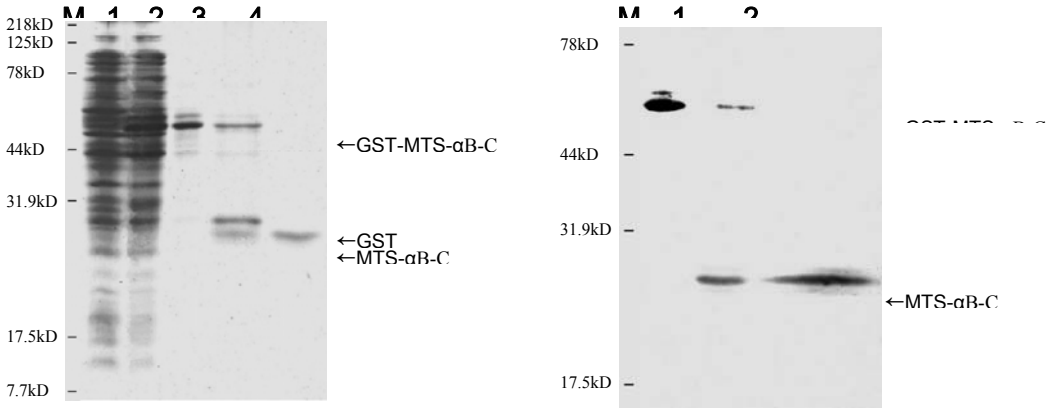


Fig.1. Construction of the bacterial expression vector pGEX-MTS-αB-C.
A, Construction scheme of the bacterial expression vector pGEX-MTS-alpha B-C. B, Diagnostic EcoRI digestion of pGEX-MTS and recombinant pGEX-MTS-alpha B-C plasmids. Lane1: 1kb ladder. Lane2: pGEX-MTS-alpha B-C. Lane3: EcoRI-digested pGEX-MTS-alpha B-C. Lane4: pGEX-MTS. Lane5: EcoRI-digested pGEX-MTS.

Expression and purification of GST-MTS- alpha B-C and MTS- alpha B-C

Fig.2 contains SDS-PAGE (A) and Wesern immunoblot analysis (B) of the expression and purification of recombinant MTS- alpha B-C. Fig.2A is a Coomassie Blue stain of a polyacrylamide electrophoretic gel run in the presence of 0.1% SDS. Control of induction of GST- MTS- alpha B-C fusion protein expression was apparent in crude cell lysates of bacterial cultures transformed with the expression plasmid pGEX- MTS- alpha B-C before and after

the addition of isopropyl-beta -D-thiogalactopyranoside (IPTG) (Fig.2A, lanes1 and 2,respectively).Treatment of the affinity-isolated fusion protein (Fig.2A, lane3) with the serine protease Factor Xa demonstrated that this fusion protein was cleaved into two distinct polypeptides (Fig.2A, lane4) that migrate to molecular weights corresponding to native GST (26kD) and native MTS plus alpha B-C (23kD). Although preparations of GST-MTS- alpha B-C fusion protein still contained a few contaminating proteins(Fig.2A, lanes 3 and 4), the recombinant MTS-alpha B-C was purified to electrophoretic homogeneity by anion exchange chromatography in the presence of 8M urea as assessed by SDS-PAGE(Fig.2A, lane5). Further confirmation of the expression and purification of recombinant MTS- alpha B-C was demonstrated by Western immunoblot analysis using anti-human alpha B-C antibody (Fig.2B). One predominant immunoreactive band was observed in the isolated uncleaved fusion protein (Fig.2B, lane1), in the mixture of cleaved fusion protein (Fig.2B, lane2), and in purified MTS- alpha B-C (Fig.2B, lane3).



A **B**

Fig.2. Expression and purification of recombinant MTS-alpha B-C in E.coli.
A, SDS-PAGE analysis of the expression and purification of MTS-alpha B-C. A Coomassie Blue stain is shown. Lanes1 and 2: crude cell lysates of bacterial cultures transformed with pGEX-MTS-alpha B-C before and after induction with 1mmol/L IPTG,respectively. Lane3: affinity-isolated GST-MTS-alpha B-C fusion protein(4μg).Lane4: Factor Xa-cleaved GST-MTS-alpha B-C fusion protein(4μg). Lane5: Purified recombinant MTS-alpha B-C(1μg). B, Western immunoblot of recombinant MTS-alpha B-C. Proteins were SDS-electrophorisized and then transferred to a nitrocellulose membrane and reacted with human alpha B-C antibody. Shown is a horseradish peroxidase development of bound MTS-alpha B-C-IgG complexes. Lane 1: GST-MTS-alpha B-C fusion protein. Lane 2: Factor Xa-cleaved GST-MTS-alpha B-C fusion protein. Lane 3: Purified recombinant MTS-alpha B-C. M: kaleidoscope prestained standards(Bio-Rad).

Disaggregation effect of MTS- alpha B-C on denatured proteins

In normal state, actins existing in myocardial homogenate as soluble were all present in the supernatant(Fig.3A, NC: S and P). Treatment of myocardial homogenate with 200mmol/L H_2O_2 resulted in precipitation of most proteins, very few actins left in the supernatant (Fig.3A, H_2O_2: S and P) measured by Western immunoblot using anti-mouse actin antibody. After the precipitates induced by 200mmol/L H_2O_2 were incubated with MTS- alpha B-C, HSP25 and ATP-containing buffer only at 37℃ for 4 hours, the actins of which in the presence of MTS- alpha B-C or HSP25 were most disaggregated and resolubilized once again to the supernatant (Fig.3B, lanes1 and 2), while the actins of which using only ATP-containing buffer were not in the supernatant (Fig.3B, lane3). It was estimated that molecular chaperone-like activity of MTS- alpha B-C is equivalent to that of HSP25 by density of the band of the disaggregated actin.

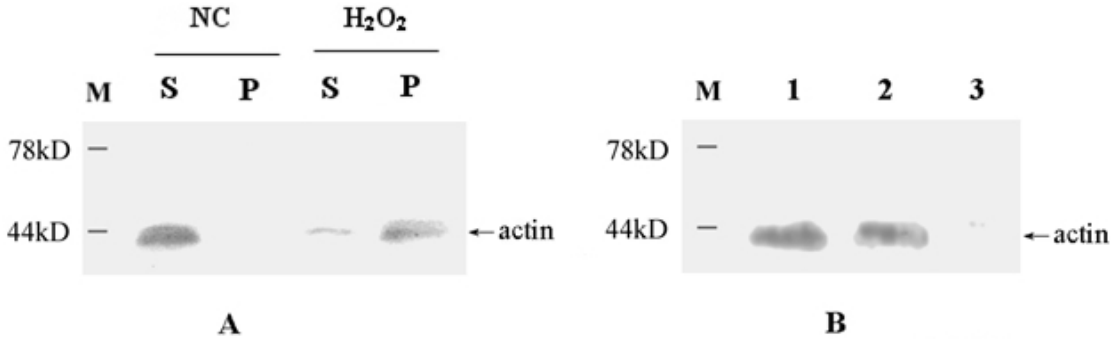


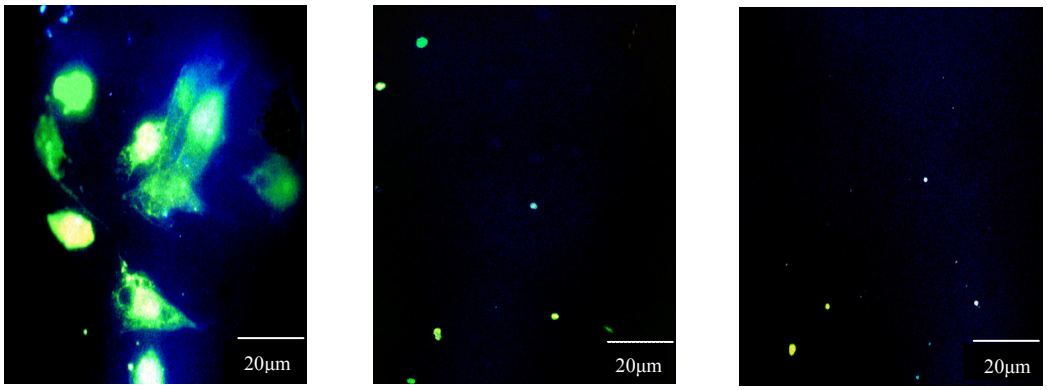
Fig.3 In vitro determination of chaperone like activity of purified MTS-alpha B-C.

A, H_2O_2 (200mmol/L) resulted in precipitation of myocardial homogenate. Only actin of which was analyzed by Western immunoblot. NC was short for normal control; H_2O_2 denoted H_2O_2 –induced myocardial homogenate. In both NC and H_2O_2, S denoted the supernatant and P denoting the precipitate. (B) MTS-alpha B-C mediated disaggregation of actin precipitates induced by H_2O_2. After precipitates induced by H_2O_2 were incubated with buffers containing MTS- alpha B-C(lane 1) or HSP25(lane 2) or with buffer only(lane 3) , the supernatants of the reaction mixtures were used for analysis of the disaggregated actin by Western immunoblot . M: kaleidoscope prestained standards(Bio-Rad).

Fluorescein isothiocyanate labeling of MTS- alpha B-C and cardiomyocytic import of FITC- MTS- alpha B-C

Under given conditions, the ratios of MTS- alpha B-C, alpha B-C and BSA to labeled fluorescein isothiocyanate (FITC μg/protein mg) respectively are: 43.4, 41.7, 25.0. This is on average equivalent to 2.8mol FITC/mol MTS- alpha B-C, 2.4mol FITC/mol alpha B-C, 4.3mol FITC/mol BSA. After confluent Neonatal rat cardiomyocytes were incubated with FITC-labeled MTS- alpha B-C, FITC-alpha B-C and FITC-BSA respectively at 37 ℃ for 8 hours, large amounts of

FITC-labeled MTS- alpha B-C entered the cells (Fig.4A) while FITC-labeled alpha B-C and BSA all did not (Fig.4B and C).



A **B** **C**

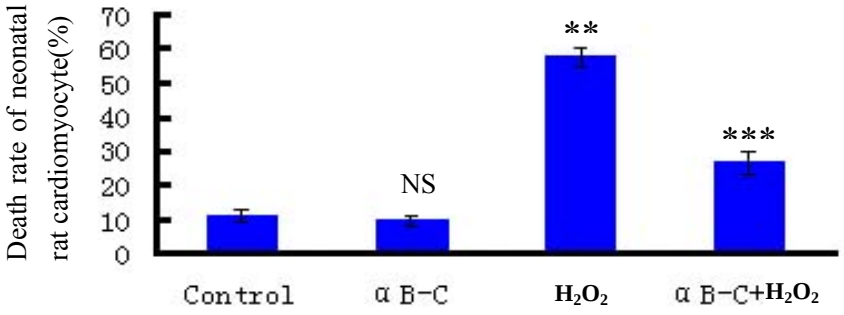
Fig.4 Cardiomyocytic import of MTS- alpha B-C.
Fluorescence microscopy of neonatal rat cardiomyocytes incubated with FITC-labeled MTS-alpha B-C(A), alpha B-C(B) and BSA(C) (40x10).

Protective effect of recombinant MTS-alpha B-C on neonatal rat cardiomyocytes

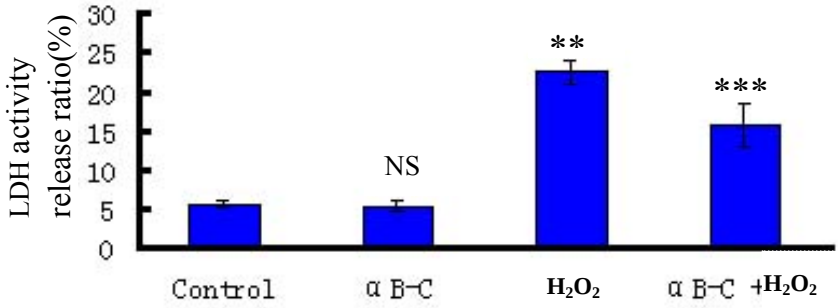
The results of experiments determining the cell death rate using Trypan Blue and the LDH release ratio are shown in Fig.5. As shown in Fig.5A,the death rate of normal neonatal rat cardiomyocytes was 11±1.6%(n=6),and it was 9.6%±1.2%(n=6) in MTS- Alpha B-C-treated cells. After incubation with 0.5mM H_2O_2, the cell death rate in untreated cells was 58±2.5%(n=6), but it was 26.8±3.3%(n=6) in MTS-alpha B-C-treated cells. The death rate in MTS-alpha B-C-treated cells was not higher than that in normal neonatal rat cardiomyocytes($p>0.05$, 11% vs 9.6%), which seemed to indicate that MTS-alpha B-C import is not cytotoxic. After incubation with 0.5mmol/L H_2O_2, the death rates in both MTS- alpha B-C-treated and untreated cells were greater than that in normal cells, which suggested that 0.5 mmol/L H_2O_2 had greatly damaged the cultured cells and that the imported MTS- alpha B-C did develop a cytoprotective effect against injury induced by H_2O_2.

As shown in Fig.5B, the ratio of LDH activity in medium to that in cells (LDH activity release ratio) in normal neonatal rat cardiomyocytes was 5.6±0.55%(n = 6), and it was 5.4±0.66%(n = 6) in MTS- alpha B-C-treated cells. After incubation with 0.5mM H_2O_2, the LDH release ratio in untreated cells was22.46±1.54%(n = 6), but it was15.67±2.75%(n = 6) in MTS- alpha B-C-treated cells. Consistent with above result of the cell death rate, the release ratio in MTS- alpha B-C-treated cells was not higher than that in normal neonatal rat cardiomyocytes either($p>0.05$, 5.4% vs 5.6%), which also indicated that MTS- alpha B-C import is not cytotoxic. After incubation with 0.5mmol/L H_2O_2, the release ratios in both MTS- alpha B-C-treated and

untreated cells were greater than that in normal cells($p<0.01$, 15.67.8% vs 5.6% and 22.46% vs 5.6%, respetively), which also suggested that 0.5mmol/L H_2O_2 had greatly damaged the cultured cells; but the ratio in MTS- alpha B-C-treated cardiomyocytes was lower than that in untreated cells($p<0.01$, 15.67% vs 22.46%), suggesting that the imported MTS- alpha B-C did develop a cytoprotective effect against injury induced by H_2O_2.



A



B

Fig.5 Protective effects of MTS- alpha B-C on neonatal cardiomyocytes against injury induced by hydrogen peroxide.

A. Death rate of neonatal rat cardiomyocytes by the dye staining. NSp>0.05 vs control; **p<0.01 vs control; ***p<0.01 vs H_2O_2 treated cells. B. Percent release of lactate dehydrogenase (LDH) from non-treated or treated cultured neonatal rat cardiomyocytes.NSp>0.05 vs control;**p<0.01 vs control; ***p<0.01 vs H_2O_2 treated cells. Data are mean ±sd, n=6.

DISCUSSION

The protective effect of HSPs on cells has early been confirmed(Xiao et al. 1999; Li et al. 1982; Radford et al. 1996; Martin et al. 1997).On the other hand, studies have shown that the mutations of alpha -cystallins causes a desminopathy, results in cardiac hypertrophy and diminishes the protective ability against stress-induced lens epithelial cell apoptosis(Wang et al. 2001; Andley et al. 2002). As a small heat shock protein,

alpha B-C is an intracellular protein in cardiomyocytes, which can be up-regulated obviously under stress conditions to protect cells against injury[25], but stress conditions themselves are harmful to cells as well. Therefore, it will be very significant that recombinant alpha B-C can be delivered into living cells in an untoxic manner, raising the levels of alpha B-C in cells so that during a given time, it can exert a cytoprotective effect, especially in clinical practice.

Considering limits of typical intracellular delivery methods such as gene transformation, liposome package and microinjection, we fused MTS into alpha B-C, hoping the entry of alpha B-C into cells by MTS mediation. Our results suggested that MTS-alpha B-C fusion protein indeed entered the cardiomyocytes, but other proteins including alpha B-C and BSA without MTS did not. This not only excluded an unspecific diffusion of the molecules due to membrane damage and passive leakage, but also suggested that it was MTS that mediated the import activity of MTS-alpha B-C.

We also investigated the effects of different concentrations and different incubation times of MTS-alpha B-C on the import. We found that when the concentration of MTS-alpha B-C was reduced to 3.0 μ M, a lot of it still got into the cells. On the contrary, when BSA was increased to 90μM, it did not enter the cells either (data not shown). All these suggested that the import activity of MTS is effecient. When FITC-labeled MTS-alpha B-C was incubated with cardiomyocytes at 37℃, we did not find the entry of the protein into the cells under fluorenscence microscope until for 4h. The import rate in this result was slower than that reported previously[12], the mechanism of which is unknown, perhaps due to the fact that MTS-alpha B-C assembles into large oligomers, and it needs to be elucidated in the future experiments.

With an in vitro experiment, we demonstrated that MTS-alpha B-C had chaperone-like function, which is consistent with previous reports(Horwitz. 1992). The fact that the MTS, even the GST-MTS at the N-terminus of the alpha B-C showed little impairment on the chaperone-like activity of alpha B-C (data not shown) further suggested that well conserved C-terminal domain of the alpha B-C is responsible for its chaperone-like function.

To estimate cytotoxicity of the protein import, we first stained MTS-alpha B-C-treated or –untreated cells with Trypan Blue in order to make sure the percentage of viable cells. It was found there was no significant difference of the cell death rate between control and MTS-alpha B-C-treated cardiomyocytes(Fig.5A, $p>0.05$, 11% vs 9.6%). Furthermore, we decided whether or not the protein import possesses cytotoxicity by determining the ratio of LDH release from cells to LDH in cells. As a result, no marked change of the LDH release ratio was observed in MTS-alpha B-C treated cells compared with that in control(Fig.5A, $p>0.05$, 5.4% vs 5.6%). All these results indicate that the protein import is not cytotoxic; at least within this concentration range, similar to previous report(Rojas et al.

1998; Eaton et al. 2000).

After treatment with 0.5mM H_2O_2 for 3h of the neonatal rat cardiomyocytes, by Trypan Blue staining, the death rate went up to 58% in H_2O_2-treated cells from 11% in control, and the percentage of LDH release ratio went up to 22.46% in H_2O_2-treated cells from 5.6% in control(Fig.5B, $p<0.01$),suggesting that 0.5mM H_2O_2 had greatly damaged cultured neonatal rat cardiomyocytes. In contrast, after treatment with 0.5mM H_2O_2 for 3h of MTS-alpha B-C treated neonatal rat cardiomyocytes, the cell death went down to 26.8% in MTS-alpha B-C treated cells from 58% in untreated cells(Fig.5A, $p<0.01$) by Trypan Blue staining, and the percentages of LDH release ratio went down to 15.7% in MTS-alpha B-C treated cells from 22.5% in untreated cells(Fig.5B, $p<0.01$). The experiments were repeated three times. For each time the experimental result had the similar tendency toward protection of cardiomyocytes against injury induced by H_2O_2, perhaps through the mechanism by which alpha B-C binds both desmin and cytoplasmic actin to maintain the structural integrity of myocytes(Golenhofen et al. 1998; Wang et al. 1996; Djabali et al. 1997; Arai et al. 1997) .All these results indicated that the imported MTS-alpha B-C did develop a protective effect on the cells against oxidative stress damage.

The import mechanism of MTS remains unclear(Schwarze et al. 2000; Gariépy et al. 2001). Protein import occurred at 22 ℃ and 37 ℃, but the import activity was greatly impaired at 4 ℃, so it is inferred that cell membrane fluidity and membrane protein mobility are important for protein import; in addition, the imported protein does not enter the intracellular compartment where intracellular proteases are highly active such as lisosomes, and retains a substantial degree of native conformation, so that the imported protein can show the physiological function in cells(Rojas et al. 1998; Ray et al. 2001). This result reported here showed the increased H_2O_2-tolerence of MTS-alpha B-C-treated cells, demonstrating that MTS-alpha B-C within cells possesses chaperone-like biological function.

In addition, future experiments will be needed to determine whether MTS-alpha B-C enters cells in oligomers or in monomers that disaggregate from oligomers that may form equilibrium between predominant oligomers and minor monomers, since in vitro MTS-alpha B-Cs form oligomers in a buffer solution(data not shown).

In spite of unknown mechanism of delivery, the fact that MTS-alpha B-C can be delivered into living cells to improve cytoprotection and exert effects on life processes within the cells is encouraging, although many problems must be solved, such as the import efficiency, immunogenesis and targeted import of alpha B-C before alpha B-C is used for clinical cardiovascular protection.

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REFERENCES

Andley UP, Patel HC, Xi JH. The R116C mutation in alpha A-crystallin diminishes its protective ability against strees-induced lens epithelial cell apoptosis. J Biol Chem. 2002; 277: 10178-10186.

Arai H, Atomi Y. Chaperone activity of alpha B-crystallin suppresses tubulin aggregation through complex formation. Cell Struct Funct. 1997; 22: 539-544.

Aoi M, Date I, Tomita S, Ohmoto T. Single or continuous injection of glial cell line-derived neurotrophic factor in the striatum induces recovery of the nigrostriatal dopaminergic system. Neurol. Res. 2000; 22: 832-836.

Beijing Medical College. Experimental immunology. People Health Press, Beijing, P.R.China, 1980; pp 203-214.

Djabali K, de Nechaud B, Landon F, Portier MM. Alpha B-crystallin interacts with intermediate filaments in response to stress. J Cell Sci. 1997; 110: 2759-2769.

Eaton P, Awad WI, Miller JI, Hearse DJ, Shattock MJ. Ischemia preconditioning: a potential role for constitutive low molecular weight stress protein translocation and phosphorylation ? J Mol Cell Cardiol. 2000; 32: 961-971.

Gariépy J, Kawamura K. Vectorial delivery of macromolecules into cells using peptide-based vehicles. TRENDS in Biotechnol. 2001; 19: 21-28.

Ghosh JG, Estrada MR, Houck SA, Clark JI. The function of the β3 interactive domain in the small heat shock protein and molecular chaperone, human αB crystalline.Cell Stress & chaperone. 2006; 11(2): 187-197.

Ghosh JG, Estrada MR, Clark JI. Interactive domain for chaperone activity in the small heat shock protein, humanαB-crystallin . Biochemisry 2005; 44: 14854-14869.

Golenhofen N, Ness W, Koob R, Htun P, Schaper W, Drenckhahn D. Ischemia-reduced phosphorylation and translocation of stress protein alpha B-crystallin to Z lines of myocardium. Am J Physiol. 1998; 274: H1457-1464.

Golenhofen N, Htun P, Ness W, Koob R, Schaper W, Drenckhahn D. Binding of the stress protein alpha B crystallin to cardiac myofibrils correlates with the degree of myocardial damage during ischemia/reperfusion in vivo. J Mol Cell Cardiol. 1999; 31: 569-580.

Goloubinoff P, Mogk A, Ben Zvi AP, Tomoyasu T, Bukau B. Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Pro. Natl. Acad. Sci. USA 1999; 96: 13732-13737.

Horwitz J. Alpha -crystallin can function

as a molecular chaperone. Proc Natl Acad Sci USA 1992; 89: 10449-10453.

Indraccolo S, Morini M, Gola E, Carrozzino F, Habeler W, Minghelli S, Santi L, Chieco-Bianchi L, Cao Y, Albini A, Noonan DM. Effects of angiostatin gene transfer on functional properties and in vivo growth of kaposi's sarcoma cells. Cancer Res. 2001; 61: 5441-5446.

Iwata A, Sai S, Nitta Y, Chen M, de Fries-Hallstrand R, Dalesandro J, Thomas R, Allen MD. Liposome-mediated gene transfection of endothelial nitric oxide synthase reduces endothelial activation and leukocyte infiltration in transplanted hearts. Circulation 2001; 103: 2753-2759.

Kato K, Shinohara H, Kurobe N ,Inaguma Y, Shimizu K, Ohshima K. Tissue distribution and developmental profiles of immunoreactive alpha B crystallin in the rat determined with a sensitive immunoassay system. Biochim Biophys Acta 1991; 1074: 201-208.

Laemmli UK. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.

Li GC, Werb Z. Correlation between synthesis of heat shock protein and development of thermotolerance in Chinese hamster fibroblasts. Proc Natl Acad Sci USA 1982; 79: 3218-3222.

Martin JL, Mestril R, Hilal-Dandan R, Brunton LL, Dillmann WH. Small heat shock proteins and protection against ischemia injury in cardiac myocytes. Circulation 1997; 96: 4343-4348.

Melkani GC, Cammarato A, Bernstein SI. αB-crystallin maintains skeletal muscle myosin enzymatic activity and prevents its aggregation under heat-shock stress. J. Mol. Biol. 2006; 358: 635-645.

Muchowski PJ, Bassuk JA, Lubsen NH, Clark JI. Human alpha B-crystallin, small heat shock protein and molecular chaperone. J.Biol.Chem. 1997; 272: 2578-2582.

Nefti O, Grongnet JF, David JC. Overexpression of αB crystalline in the gastrointestinal tract of the newborn piglet after hypoxia. Shock. 2005; 24(5): 455-461.

Radford NB, Fina M, Benjamin IJ, Moreadith RW, Graves KH, Zhao P, Gavva S, Wiethoff A, Sherry AD, Malloy CR, Williams RS. Cardioprotective effects of 70-kDa heat shock protein transgenic mice. Proc Natl Acad Sci USA 1996; 93: 2339-2342.

Ray PS, Martin JL, Swanson EA, Otani H, Dillmann WH, Das DK. Transgene overexpression of alpha B crystallin confers simultaneous protection against cardiomyocyte apoptosis and necrosis during myocardial ischemia and reperfusion. FASEB J. 2001; 15: 393-402.

Rojas M, Donahue JP, Tan ZJ, Lin YZ. Genetic engineering of protein with cell membrane permeability. Nat Biotechnol. 1998; 16: 370-375.

Schwarze SR, Dowdy SF. In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. Tips. 2000; 21: 45-48.

Simons PC , Vander Jagt DL. Purification of glutathione S-transferases from human liver by glutathione-affinity chromatography. Anal.Biochem. 1977; 82: 334-341.

Smith D B, Johnson K S. Single-step Purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-tranferase. Gene 1988; 67: 31-40.

Wang K, Spector A. Alpha-crystallin stabilizes actin filaments and prevents cytochalasin-induced depolymerization in a phosphorylation-dependent manner. Eur J Biochem. 1996; 242: 56-66.

Wang XJ, Osinska H, Klevitsky R, Gerdes AM, Nieman m, Lorenz J, Hewett T, Robbins J. Expression of R120G-alpha B-crystallin causes aberrant desmin and alpha B-crystallin aggregation and cardiomyopathy in mice. Circ Res. 2001; 89: 84-91.

Xiao X Z, Benjamin K S. Human genetics'99: The cardiovascular system stress-response proteins in cardiovascular disease. Am J Hum Genet. 1999; 64: 685-690.