Biochemical and Biophysical Research Communications 446 (2014) 1250–1254

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Neuronal cells but not muscle cells are resistant to oxidative stress mediated protein misfolding and cell death: Role of molecular chaperones

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ARTICLE INFO

Article history:
Received 9 March 2014
Available online 28 March 2014

Keywords:
Protein misfolding
Heat shock proteins
Oxidative stress
Skeletal muscle
Neuron
Cell viability

ABSTRACT

Our recent study in a mouse model of familial-Amyotrophic Lateral Sclerosis (f-ALS) revealed that muscle proteins are equally sensitive to misfolding as spinal cord proteins despite the presence of low mutant CuZn-superoxide dismutase, which is considered to be the key toxic element for initiation and progression of f-ALS. More importantly, we observed differential level of heat shock proteins (Hsp's) between skeletal muscle and spinal cord tissues prior to the onset and during disease progression; spinal cord maintains significantly higher level of Hsp's compared to skeletal muscle. In this study, we report two important observations; (i) muscle cells (but not neuronal cells) are extremely vulnerable to protein misfolding and cell death during challenge with oxidative stress and (ii) muscle cells fail to mount Hsp's during challenge unlike neuronal cells. These two findings can possibly explain why muscle atrophy precedes the death of motor neurons in f-ALS mice.

1. Introduction

Protein misfolding and aggregation are considered as hallmark for number of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS) [1]. In order to maintain the functional structure of proteins and enzymes, several strategies have evolved including the molecular chaperone network to respond to chronic cellular stress. Molecular chaperones namely heat shock proteins (Hsp's) act as catalysts for proper folding of misfolded or unfolded proteins [1]. The importance of Hsp's in regulation of protein misfolding and aggregation in neurodegenerative diseases is demonstrated by observations that (i) overexpression of selective Hsp's delays the progression of disease in animal models of neurodegeneration [1–3] and (ii) Hsp's are colocalized with aggregated proteins in diseases such as AD and PD [1]. Moreover, Hsp's have recently been shown to trigger the solubilization of toxic protein aggregates [1]. Together, these findings demonstrate the essential role of Hsp's in the regulation of proteostasis in the central nervous system (CNS). In comparison, nothing is known about protein misfolding and its regulation by Hsp's in skeletal muscle although several studies in the literature demonstrate that selective overexpression of Hsp's in cultured cells and intact animals reduce myotube/muscle atrophy [4–7]. Studies from our group have consistently shown that misfolding of skeletal muscle cytosolic proteins is a consistent finding in mouse models that exhibit significant muscle atrophy [8–10].

While recently studying protein misfolding in spinal cord and skeletal muscle (two tissues primarily affected in ALS) from wild-type (WT) mice and G93A mouse model of familial-ALS (f-ALS), we made an interesting observation: the expression of all the Hsp's studied (40, 60, 70 and 90) were strikingly lower in skeletal muscle versus spinal cord in the same WT animals [11]. This observation would suggest that there is less likelihood for the repair of misfolded proteins in skeletal muscle versus spinal cord and could possibility translate into increased susceptibility of muscle cells to oxidative stress/damage mediated cell death versus cells present

Abbreviations: f-ALS, familial-Amyotrophic Lateral Sclerosis; BisANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt; Hsp, heat shock protein; tBHP, *tert*-butyl hydroperoxide.

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http://dx.doi.org/10.1016/j.bbrc.2014.03.097
0006-291X/

in the CNS such as neurons. Therefore, in this study we asked (i) if muscle cells are more vulnerable to oxidative stress induced misfolding and cell death, compared to neuronal cells, (ii) if these findings are linked to the differential response of protective molecular chaperones. Here we first show that skeletal muscle cytosolic proteins from WT mice show a higher kinetic of protein misfolding in response to *in vitro* oxidative and heat stress, compared to spinal cord cytosolic proteins. Next we show that cultured muscle cells are highly susceptible to oxidative stress mediated protein misfolding and cell death compared to neuronal cells, which is intimately linked to their inability to induce a Hsp response, unlike in neuronal cells. Together, these findings suggest for the first time that differential level of Hsp's and their response are possible determinants for the integrity of neuronal cells (versus muscle cells) in the event of an oxidative challenge.

2. Materials and methods

2.1. Animals and cell lines

4-mo-old C57BL/6 WT mice were used for measuring the expression of Hsp's and *in vitro* cell free-system assay. All procedures for handling animals were reviewed and approved by the Institutional Animal Care and Use Committee of University of Texas Health Science Center at San Antonio. 88NPC cells were grown with N-2 medium (containing DMEM/F-12, penicillin, gentamicin, B27, transferring, insulin and βFGF) in Matrigel-coated 100 mm dishes. C2C12 cells were grown in 100 mm dishes with DMEM high glucose + 20% FBS + 100 U/ml Penicillin + 100 μg/ml Streptomycin.

2.2. Measurement of change in protein surface hydrophobicity in a cell-free system

Skeletal muscle and spinal cord tissues from 4-mo-old WT mice were homogenized in buffer containing 50 mM Tris, pH 7.4 buffer containing 1 mM $MgSO_4$ and protease cocktail inhibitors followed by centrifugation at 100,000*g* for 1 h at 4 °C. Cytosolic proteins (100 μg) were incubated for (i) 1 h in *tert*-butyl hydroperoxide (tBHP) at 37 °C or for (ii) 1 h at 25 °C, 37 °C, and 42 °C. After the incubation, a reaction mixture containing 50 μg (1 mg protein/ml) proteins was set up for photo-labeling under UV light with 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt (BisANS, 0.1 mM) as described earlier [8,10]. Equal amounts of BisANS-labeled proteins (10 μg) were subjected to 12% SDS–PAGE and BisANS fluorescence captured under 365 nm UV light with an AlphaImage™ 3400 and followed by overnight staining of gels in SYPRO Ruby to normalize for protein loading. SYPRO Ruby fluorescence was captured on a Typhoon 9400 Variable Mode Imager (Amersham Biosciences) under the settings 610 BP 30, 520 PMT, 532 nm.

2.3. Measurement of change in protein surface hydrophobicity in cultured cells

Cells were stressed with 0, 50, 100, and 200 μM tBHP for 12 h in serum-free medium supplemented with 2% BSA. Cells were washed in ice-cold PBS, harvested in 50 mM Tris, pH 7.4 buffer containing 1 mM $MgSO_4$, protease cocktail inhibitors and centrifuged at 100,000*g* for 1 h at 4 °C. A reaction mixture of 50 μg (1 mg protein/ml) proteins with BisANS (0.1 mM) was set up for photo-labeling under UV light as described earlier [8,10]. Equal amounts of BisANS-labeled proteins (10 μg) were subject to 12% SDS–PAGE, BisANS fluorescence captured and normalized to SYPRO Ruby as described above.

2.4. Heat shock proteins in 88NPC and C2C12 cells

Cells were stressed with 0, 50, 100, and 200 μM tBHP for 12 h in serum-free medium supplemented with 2% BSA. Cells were washed in ice-cold PBS and harvested in 20 mM potassium phosphate buffer pH 7.4 containing 0.5 mM $MgCl_2$, 1 mM EDTA and protease inhibitors. Cells were sonicated and centrifuged at 100,000*g* for 1 h at 4 °C and equal amount of proteins (20 μg) were subject to 12% SDS–PAGE followed by western blot using anti-rabbit primary antibodies against Hsp-60 and -70, and β-Actin (loading control). HRP secondary antibodies were used to visualize the distribution of hsp's.

2.5. 88NPC and C2C12 WST-1 cell viability

Cells were plated into a 96-well plate at 30,000 cells/well in serum-free medium supplemented with 2% BSA and allowed to recover and attach for 24 h. Thereafter, cells were stressed with 0, 50, 100, and 200 μM tBHP for 12 h in serum-free medium supplemented with 2% BSA. Upon completion of tBHP incubation, the plates were spun down at 1000*g* for 2 min and old medium was aspirated. 100 μl of fresh serum-free medium supplemented with 2% BSA and 5 μl of WST-1 reagent was added to each well and incubated at 37 °C for 3.5 h. The absorbance at 450–630 nm was read on a microplate reader.

3. Results

Our recent report showed that at any given time point, the expression of molecular chaperones, namely Hsp's differs considerably between spinal cord and skeletal muscle tissues from WT and G93A mouse model of familial-ALS [11]. The expression of all the Hsp's (Hsp-40, -60, -70 and -90) tested were significantly higher in spinal cord versus skeletal muscle [11]. We hypothesized that this dramatic difference in molecular chaperones could possibly explain the surprising finding that protein misfolding pattern is quite similar between spinal cord and muscle tissues of G93A mice, despite considerable difference in the expression of the mutant G93A protein between these tissues [11]. Because G93A overexpression in mice is known to increase endogenous oxidative stress [12–14] and level of Hsp's differ between spinal cord and skeletal muscle, here we first asked if spinal cord proteins in general are more resistant to stress induced misfolding, compared to skeletal muscle proteins in an *in vitro* assay in cytosolic protein extracts. We used 4-mo-old WT mice to perform these experiments that exhibit ~15- and 5-fold higher expression of Hsp-70 and Hsp-60, respectively, in spinal cord versus skeletal muscle tissues (Fig. 1A and B). Using the BisANS photo-labeling approach, we found that in response to oxidative stress (tBHP), the kinetics of hydrophobic domain exposure is slower in spinal cord proteins compared to skeletal muscle proteins (Fig. 2A and B). In response to heat shock stress, the difference between spinal cord and skeletal muscle proteins with respect to the kinetics of hydrophobic domain exposure is even more dramatic. Skeletal muscle proteins progressively lost their hydrophobic pockets with increasing temperature (~60% decrease at 42 °C compared to 25 °C), whereas, spinal cords proteins were completely resistant to unfolding (Fig. 2C and D). These results clearly indicate that the cellular level of Hsp's could be a critical component in our findings as the assays were performed in cytosolic extracts prepared in the presence of protease inhibitors, thus ruling out the effect of proteasome. Although our findings were interesting, we still didn't know if they translated into any functional consequence. To address this question, we first determined the global status of protein folding in C2C12 myoblasts and NPCs exposed to varying concentrations of tBHP. The data in

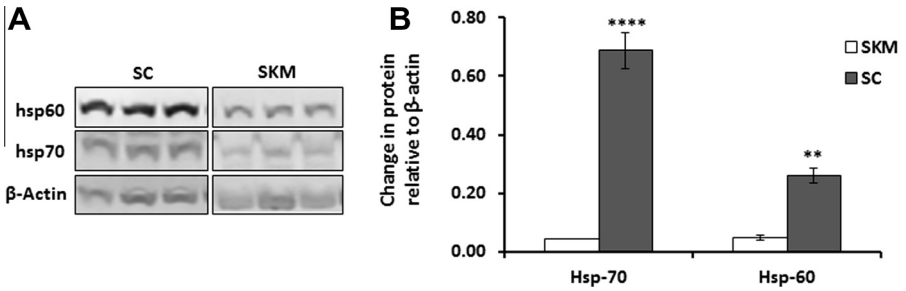

Fig. 1. Low level of protective chaperones in skeletal muscle compared to spinal cord. (A) Heat shock proteins (Hsp's)-60 and -70 were detected by immunoblot in cytosolic fractions from skeletal muscle and spinal cord of 4-mo-old wild-type (WT) C57BL/6 mice; (B) quantification of immunoblots in (A). Results are expressed as mean ± SEM of 3 samples and analyzed by two-tailed *t*-test ($^{**}p < 0.01$ and $^{****}p < 0.0001$).

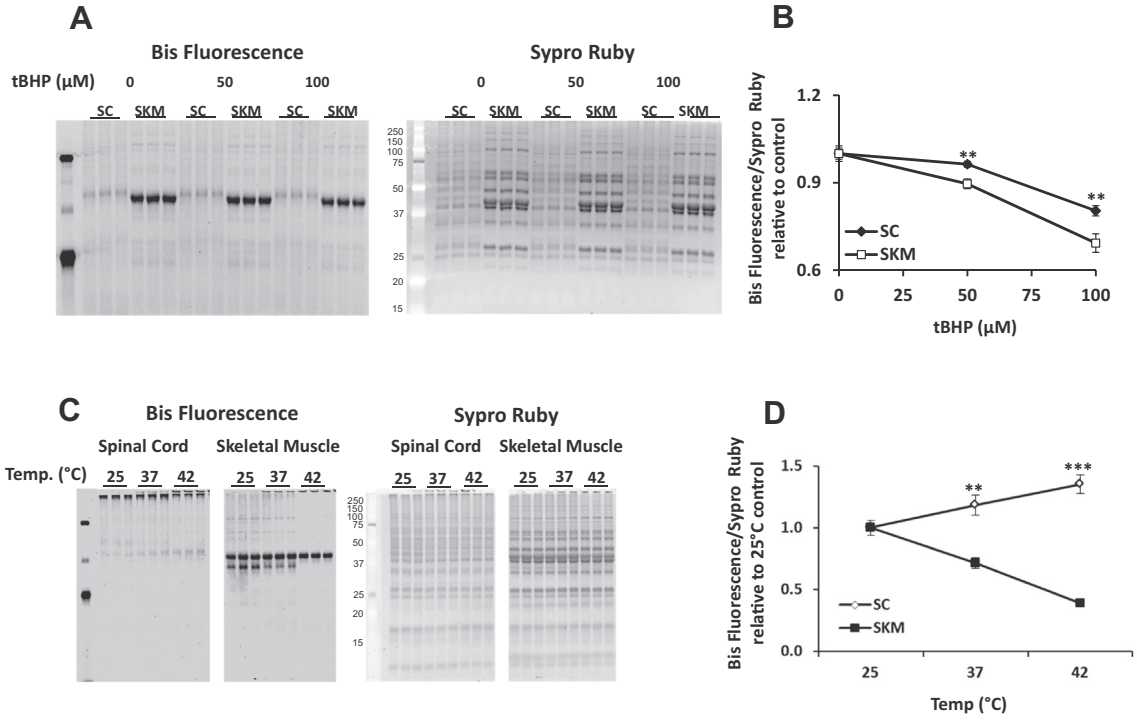

Fig. 2. Susceptibility of skeletal muscle proteins versus spinal cord proteins to heat and oxidative stress induced misfolding. Skeletal muscle and spinal cord cytosolic proteins from 4-mo-old WT C57BL/6 mice were incubated for (A) 1 h in *tert*-butyl hydroperoxide (tBHP) at 37 °C or for (C) 1 h at 25 °C, 37 °C, and 42 °C. After the incubation period, proteins were photolabeled with Bis-ANS and run on SDS–PAGE (10 μg) for visualization of BisANS labeling followed by SYPRO Ruby staining. Results (B and D) are expressed as Bis fluorescence/SYPRO Ruby staining, relative to control ($n = 3$) and analyzed by two-tailed *t*-test ($^{**}p < 0.01$ and $^{***}p < 0.001$).

Fig. 3A and B clearly demonstrates that NPC proteins are structurally stable and resistant to unfolding when exposed to different concentration of tBHP as we did not observe any marked change in BisANS incorporation, compared to proteins isolated from unstressed NPCs. On the contrary, C2C12 myoblast proteins are very sensitive to misfolding; approximately 2-fold increase in global exposure of protein hydrophobic domain was detected when cells were exposed to low concentration of tBHP (50 μM). Exposure to the highest concentration of tBHP (200 μM) caused a massive collapse of hydrophobic domain in C2C12 myoblasts. Since we observed a striking difference in protein folding status between the two cell lines in response to oxidative insult, we investigated if there is a differential chaperone response between muscle and neuronal cells. The chaperone response (Hsp-60 and -70) was found to be significantly higher in NPCs compared to C2C12 myoblasts when exposed to various concentrations of tBHP (Fig. 4A and B). C2C12 myoblasts failed to mount any Hsp response, even with the highest concentration of tBHP (Fig. 4A and B). We finally determined if these striking differences between C2C12 myoblast and NPCs with regards to protein misfolding/chaperone response translates to effect on cell viability. Our results demonstrate that myoblast cells are extremely sensitive to cell death, particularly at higher concentrations of tBHP; approximately 60% and 20% of myoblast cells were found to be viable when challenged with 100 and 200 μM of tBHP, respectively (Fig. 4C). On the contrary, 100% of neuronal cells were found to be viable even at the highest concentration of tBHP (200 μM) tested in this study (Fig. 4C).

4. Discussion

The premise for this study was an unexpected finding in our previous study which reported that protein misfolding pattern

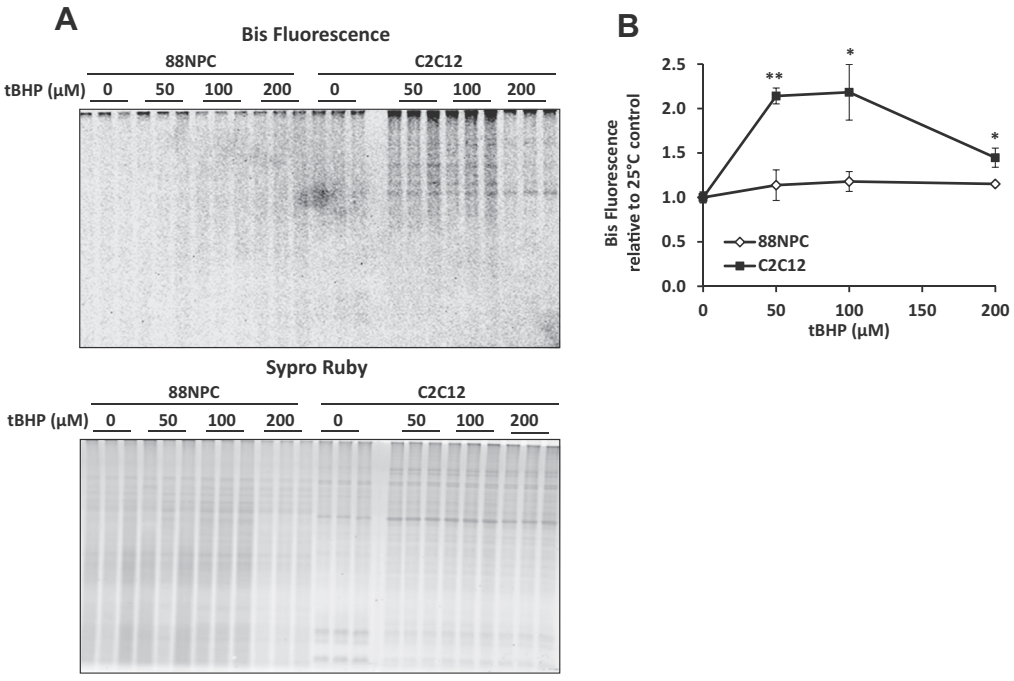

Fig. 3. Muscle cells but not neuronal cells are susceptible to oxidative stress induced misfolding. 88NPC and C2C12 cells were grown in the presence and absence of varying concentrations of *tert*-butyl hydroperoxide (tBHP) for 12 h. (A) Cytosolic proteins were isolated and photo-labeled with Bis-ANS and equal amounts of BisANS-labeled protein (10 μg) were subject to SDS–PAGE for visualization of BisANS labeling followed by SYPRO Ruby staining. Results in (B) show Bis-ANS fluorescence in tBHP treated 88NPC and C2C12 cells, relative to control untreated cells ($n = 3$). Results are expressed as mean ± SEM of 3 individual experiments and analyzed by two-tailed *t*-test ($^{*}p < 0.05$ and $^{**}p < 0.01$).

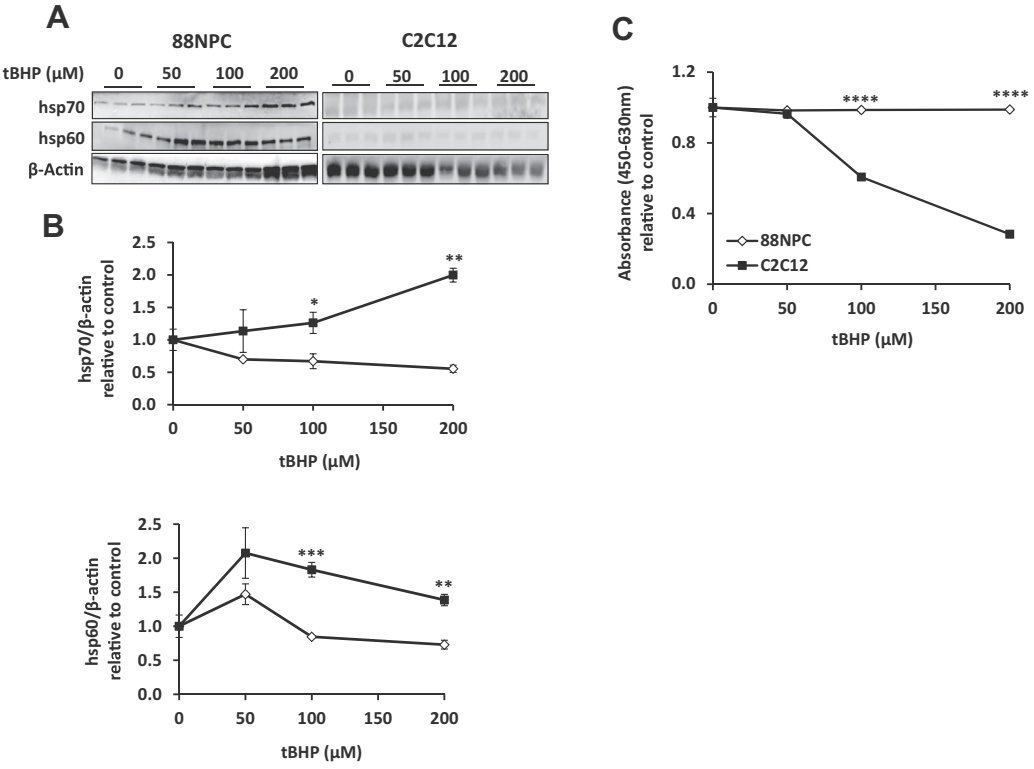

Fig. 4. Lack of chaperone response in muscle cells exposed to oxidative stress. (A) Cytosolic proteins from tBHP treated cells (12 h) were subjected to SDS–PAGE (10 μg 88NPC and 20 μg C2C12) and the expression of Hsp-60 and -70 were determined by immunoblot and expressed relative to β-Actin in (B). (C) 88NPC and C2C12 cells were grown in the presence or absence of varying concentrations of tBHP for 12 h. Upon completion of treatment, cells were supplemented with fresh media and WST-1 reagent. Cleavage of WST-1 allowed for the measurement of the absorbance at 450 nm (minus background absorbance at 630 nm), indicative of cell viability. The results are expressed as absorbance (450–630 nm), relative to control. Results are expressed as mean ± SEM of 3 individual experiments and analyzed by two-tailed *t*-test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$).

over the lifespan of the G93A mouse model of f-ALS is surprisingly similar between spinal cord and skeletal muscle tissues [11] although they differ substantially in the expression of the ALS causing mutant SOD1 (mSOD1) protein; i.e., the mSOD1 expression is significantly lower in skeletal muscle versus spinal cord tissues [11]. In addition, our *in vitro* cell-free system study in WT muscle further demonstrated the increased susceptibility of muscle proteins (compared to spinal cord proteins) to misfolding by the G93A SOD1 enzyme [11]. Our results in the study suggested that differential pattern of Hsp's between these two distinct tissues that have both been implicated in f-ALS pathology could possibly explain our findings in the G93A model of f-ALS.

The proteostasis machinery within the cells are regulated by Hsp's that repair or prevent protein misfolding and proteolytic degradation machinery regulated by proteasome that can remove oxidized and misfolded proteins [1,15,16]. Depending on the cell type, the proteostasis machinery could differ considerably in how they handle oxidized and misfolded proteins [17]. For instance, a recent study by Onesto et al. transfected G93A enzyme in both muscle and motoneuronal cells and found that muscle cells are much more efficient in clearing mSOD1 protein compared to motoneuronal cells, possibly due to higher proteasome activity [17]. Although the study did not measure the expression of Hsp's, their findings demonstrated cell specific differences in proteolytic degradation machinery. In agreement, our findings here show for the first time that while neuronal cells mount a significant chaperone response when subjected to an oxidative challenge, muscle cells completely lack this response, which can possibly explain the vulnerability of muscle cells for protein misfolding and cell death, as opposed to neuronal cells that are extremely resilient. In this study, since we did not measure proteasome activity in muscle and neuronal cells (in the presence of the oxidative stressor), it is difficult to predict how much of our effects on protein misfolding and cell death in muscle cells can be ascribed to proteasome function. However, our experiments in the cell-free system isolated from spinal cord and muscle tissues from WT mice can possibly answer this question to some extent. As we had reported earlier [11], here also we found a ~5- to 15-fold difference in basal level of Hsp's 60 and 70 between spinal cord and muscle tissues in the same young WT mice. Because our *in vitro* experiments were performed with cytosolic fractions (that were exposed to either an oxidative stressor or temperature shock) isolated in the presence of protease inhibitors to rule out the effect of proteasome, it is reasonable to conclude that a large part of the differential effects on protein misfolding and cell death that we observe between these tissues in response to an oxidative stressor is likely due to differential level of endogenous Hsp's.

In conclusion, our findings in this study demonstrates that two distinct cell types that have both been implicated in the pathogenesis of f-ALS have a differential pattern of chaperone response in the presence of an oxidative stressor, which can possibly explain why muscle atrophy precedes the death of motor neurons in the G93A mouse model of familial-ALS.

Acknowledgments

This study was supported by VA merit grant to Asish Chaudhuri and partially supported by AFAR Research Grant and National Institutes of Health (K01AG038555) grant to Arunabh Bhattacharya. We like to thank Dr. Peter Hornsby and Anuja Mishra for providing and assisting in growing the NPC's.

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