Oxidative Stress, Calcium and Age are Interdependent Regulators of the Stress Response in Duchenne Muscular Dystrophy

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Abstract

Heat shock/stress proteins (HSP) are constitutive and stress inducible proteins that are essential for survival during and after cellular stress. We previously observed the differential upregulation of HSP in hypercontracted, necrotic and regenerating muscle fibres from Duchenne muscular dystrophy (DMD) patients. Elevated levels of the 90 kD heat shock protein (hsp90) and ubiquitin in regenerating fibers were found to be primarily developmentally regulated. The present study investigates other potential mechanisms involved in the activation of the HS response in DMD. The 75 kD glucose regulated protein (grp75) and oxidation-specific stress proteins together with ferritin were employed as markers of calcium loading and oxidative stress respectively. Correlation studies were performed on the levels of these proteins and those of hsp70 and hsp60, using Western blot analysis of muscle biopsies from patients with DMD, dermatomyositis, polymyositis, and mitochondrial myopathies. Multiple regression analysis revealed a strong correlation between hsp70, hsp60, grp75, oxidation specific stress proteins, ferritin and age in DMD.

Key words: muscular dystrophy, heat shock proteins, oxidative stress, age, multiple regression.

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All organisms display a highly conserved physiological response to heat shock (HS), which involves the induction of a small set of proteins called the heat shock/stress proteins (HSP) [11]. Besides heat, a wide variety of other stresses induce the HS response including oxidative stress, and alterations in the levels of essential cellular components, e.g. calcium and glucose. The common pathway for the induction of a HS response appears to be the presence of unfolded, abnormally folded or denatured proteins [8]. While many stress inducers elicit the elevated expression of all HSP families, there are stress proteins unique to a particular agent(s). This is the case for glucose regulated proteins (GRP), oxidation-specific stress proteins as well as ferritin.

Duchenne muscular dystrophy (DMD) is a disorder for which the identification of the gene defect has not allowed full characterization of the biochemical mechanisms leading to muscle necrosis. In spite of a fixed mutation which is present from fetal life onwards, DMD is a progressive disorder, characterized by a variety of pathological processes. We have previously reported the differential upregulation of HSP in hypercontracted, necrotic and regenerating muscle fibres from DMD patients [4,5]. Investigating the regulation of the HS response in DMD may shed light on the clinical progression of the disease. Stressful stimuli that may participate in the activation of the HS response in DMD, include elevated intracellular calcium levels [16] and oxidative stress [1]. An important factor influencing the expression of stress proteins in general is age. Changes in both stress-responsiveness [14] and the kinetics of the HS response itself [7] have been related with increasing age.

In the present study we investigated the relation between the activation of the HS response, calcium perturbation, oxidative stress and age, by correlating the levels of the 70 kD and 60 kD HSP (hsp70 and hsp60), the 75 kD glucose regulated protein (grp75), oxidation-specific stress proteins, ferritin and age in patients with DMD or other mucle diseases. Multiple regression analysis, a valuable tool in determining the association between variables which has

Table1.Mean stress protein levels (SEM) in the muscle from patients with DMD, DM, PM and MM evaluated against levels in normal muscle of comparable age in 2 age ranges (N1:2-27 for DMD and DM; N2:31-68 for PM and MM).

Stress protein	Muscle					
	N1	DMD	DM	N2	PM	NN
hsp70	**521**	**908**	**1490**	**150**	**2173****	**2381****
	(174)	(369)	(494)	(15)	(664)	(899)
hsp60	**988**	**4471****	**4010***	**620**	**4098***	**4310****
	(264)	(594)	(1032)	(286)	(1741)	(1033)
grp75	**554**	**2702****	**780**	**221**	**734**	**230**
	(151)	(519)	(256)	(118)	(282)	(96)
CuZnSOD	**2550**	**3136**	**6820**	**1522**	**6336**	**4745**
	(640)	(596)	(2283)	(267)	(3137)	(1665)
MnSOD	**848**	**1937**	**5419****	**738**	**3880****	**2848***
	(89)	(158)	(1623)	(10)	(859)	(270)
GSHPx	**617**	**1897***	**3606****	**423**	**2389***	**1733**
	(196)	(309)	(958)	(37)	(365)	(125)
Catalase	**2753**	**6825**	**7169**	**1699**	**4018**	**3917**
	(882)	(1994)	(2931)	(800)	(327)	(1469)
HO	**1601**	**2122**	**3559**	**2106**	**3626**	**2205**
	(282)	(592)	(1640)	(245)	(866)	(958)
Ferritin	**752**	**1355**	**11229****	**455**	**2530**	**2682**
	(57)	(356)	(5108)	(90)	(714)	(2067)

Equal amounts of muscle protein, separated by SDS-PAGE, were analyzed by Western blotting and immunoreactive polypeptides quantified by densitometric scanning. Each point represents the mean (SEM) of seven (DMD) or four (N1, N2, PM, DM, MM) individuals. **:$p < 0.01$ and *:$p < 0.05$ in comparison with normal levels.

Table2. Stress protein levels in the muscle of individual DMD patients of different ages. The coefficients of variance (C.V.), illustrate the relative high variation in the levels of hsp70, catalase, HO and ferritin compared to the lower variation in the level s of hsp60, grp75, CuZnSOD, MnSOD and GSH Px between individuals.

Age	hsp70	hsp60	grp75	CuZnSO	DMnSO	DGSH Px	Catalase	HO	Ferritin
7	162	6661	2671	2047	2003	3038	3747	4455	1132
7	1990	4216	2079	5632	1896	2306	2175	805	703
25	598	4372	549	4387	1665	1897	5231	4190	3419
10	132	3624	1611	1975	2752	2372	5754	1309	897
8	179	2174	3947	1565	2030	1251	2372	729	1042
11	740	3883	4120	4197	1809	519	15309	1309	1452
4	2557	6369	3938	2128	1405	1897	13185	2054	839
C.V.	1.07	0.35	0.51	0.50	0.22	0.43	0.77	0.74	0.69

been used before for HSP expression [17], revealed a significant, strong correlation between the expression of hsp70, oxidation specific stress proteins (catalase, glutathione peroxidase [GSHPx]), ferritin, grp75 and age in DMD. It is proposed that hsp70 expression in DMD is mediated by free radical reactions, activated by elevated intracellular calcium and influenced by age.

Material and Methods

Patients and controls. The biological specimens selected for this study included normal and diseased muscle tissue. Normal specimens were from 2 age groups: age range: 2-27, mean age: 14, n = 4 and age range: 31-68, mean age: 44, n = 4. Abnormal muscle was obtained from patients with DMD (n = 7; mean age: 10; age range: 4-25), polymyositis (PM, n = 4; mean age: 47; age range: 34-76), dermatomyositis (DM, n = 4; mean age: 21; age range:

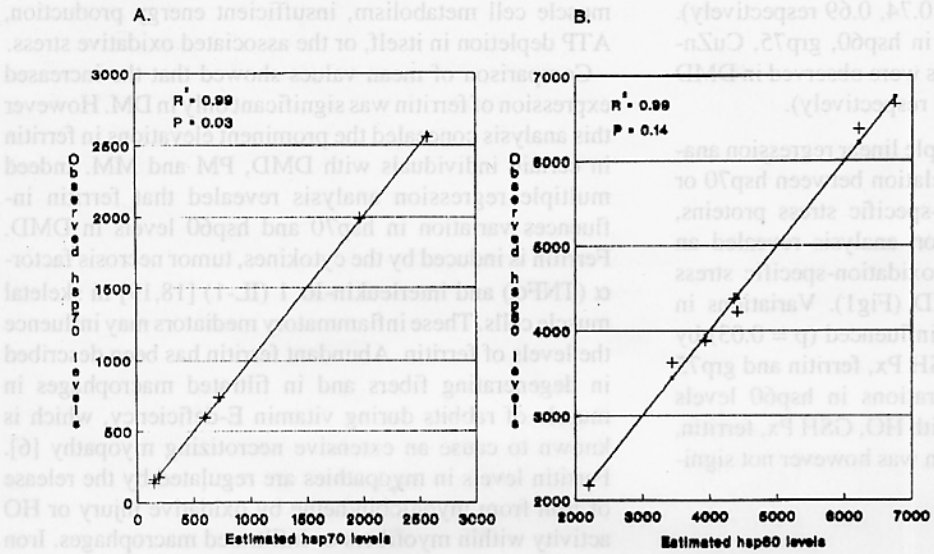

Figure 1. Multiple regression analysis. Scatterplots showing the relation between observed values (+) for hsp70 (A) and hsp60 (B) in DMD muscle and estimated values (line) calculated from multiple regression functions indicated below.
hsp70 = 24074.7 + 5.2 x 10^{-3} catalase + (-3.8)GSH Px + 7.2ferritin + (-3.6)grp75 + (-1556.8)age
hsp60 = 31361.1 + 2.0 HO + (-5.5) GSH Px + 3.8ferritin + (-4.1) grp75 + (-1423.0) age

14-38) and mitochondrial myopathies (MM, n = 4; mean age: 43; age range: 23-58). Diagnoses of these conditions were made in accordance with standard clinical, pathological, and electromyographical criteria. DMD diagnosis was confirmed by dystrophin immunohistochemistry and Western blot analysis. The protocol was approved by the Ethical Committee of the University of Pretoria and informed consent from normal controls, patients or parents was obtained in all cases. Muscle biopsies, from either the biceps or the quadriceps muscles were obtained within 15min following local or general anaesthesia. Biopsies were rapidly frozen in isopentane and stored in liquid nitrogen.

*Antibodies.*Monoclonal antibodies against hsp72 (inducible isoform of the 70kD HSP family), hsp60 and grp75 as well as a whole rabbit antiserum against rat heme oxygenase (HO) were from StressGen (Victoria, Canada). Polyclonal antibodies to human superoxide dismutases (SOD) (including copper and zinc [Cu ZnOD] and the manganese-containing enzyme [MnSOD]), GSH Px and to human liver ferritin were from Biogenesis (Bournemouth, UK). A polyclonal antiserum against human catalase was supplied by The Binding Site (Birmingham, UK). Secondary antibodies were anti mouse/rabbit Ig peroxidase-linked $F(ab')_2$ fragments from Amersham, (Buckinghamshire, UK) and anti-sheep peroxidase-conjugated IgG (H+L) from TheBinding Site (Birmingham, UK).

Western blot analysis. Equal amounts of muscle protein in SDS sample buffer, as determined according to Sheffield *et al* [15], were subjected to SDS polyacrylamide gel electrophoreses. Proteins were transferred to polyvinylidene difluoride membranes with a semi-dry transfer unit (Hoefer Scientific Instruments, San Francisco, CA, USA) at 90 mA/gel in Kyhse-Anderson buffer [10]. Blots were probed for HSP (hsp70, hsp60), grp75, antioxidant enzymes (CuZnSOD, MnSOD, GSHPx, catalase, HO) and ferritin followed by the appropriate peroxidase-linked secondary antibody, detected by chemiluminescence (Boehringer Mannheim, Germany). Scanning densitometry was used to determine relative peak areas to assess the relative amount of protein in immunoreactive bands on autoradiograms (Hoefer Scientific Instruments, SanFrancisco, CA, USA).

Statistical analysis. Analysis of variance and comparison of means were done using Statgraphics software (Statistical graphics system. Statistical Graphics Corporation, STSCInc., USA, 1986). Differences in mean values were considered significant if the least significant difference value (LSD), was exceeded, as calculated from the pooled variance. Regression analyses were done using CoStat (CoHort Software, Berkeley, CA, USA, 1990).

Results

Analysis of variance. Mean stress protein levels in whole muscle homogenates of different myopathies and normal muscle of comparable age, are summarized in Table 1. In relation to controls, hsp70 was significantly increased ($p < 0.01$) in PM and MM but not in DMD and DM, while elevated levels of hsp60 was observed in all myopathies investigated (DMD and MM: $p < 0.01$; DM and PM: $p < 0.05$). In contrast to the uniform elevation of hsp60, grp75 was exclusively upregulated in DMD ($p < 0.01$). MnSOD was significantly increased in DM ($p < 0.01$), PM ($p < 0.01$) and MM ($p < 0.05$) but not in DMD, while GSH Px was elevated in DMD ($p < 0.05$), DM ($p < 0.01$),PM ($p < 0.05$) but not in MM. The levels of CuZnSOD, catalase and HO were not influenced in any of the myopathies investigated. In spite of prominent elevations of ferritin in certain patients with DMD, PM and MM, the increase in mean ferritin values was significant only in DM ($p < 0.01$). Stress protein levels in individual DMD patients and their coefficients of variance (C.V.) together with the age of patients are presented in Table 2. The large variation in the levels of hsp70, catalase, HO and ferritin between individual DMD patients is illustrated by the relative high C.V. for

these stress proteins (1.07, 0.77, 0.74, 0.69 respectively). In contrast, moderate variations in hsp60, grp75, CuZn-SOD, MnSOD and GSH Px levels were observed in DMD (C.V.:0.35, 0.51, 0.50, 0.22, 0.43 respectively).

Regression analysis. While simple linear regression analysis showed no significant correlation between hsp70 or hsp60 and individual oxidation-specific stress proteins, grp75 or age, multiple regression analysis revealed an interrelationship between HSP, oxidation-specific stress proteins, grp75 and age in DMD (Fig1). Variations in hsp70 levels, were significantly influenced ($p = 0.03$) by age and the levels of catalase, GSH Px, ferritin and grp75 ($R^2 = 0.99$). Furthermore, alterations in hsp60 levels strongly correlated ($R^2 = 0.99$) with HO, GSH Px, ferritin, grp75 and age, but this correlation was however not significant ($p = 0.14$).

Discussion

In the present study multiple regression analysis allowed us to determine the interrelations between a number of stress factors in individual DMD patients of different ages. No simple correlation was observed between the levels of hsp70/hsp60 and oxidation-specific stress proteins, ferritin, grp75 or age. However, multiple regression analysis revealed an interdependence between hsp70 and hsp60 and oxidation-specific stress proteins, ferritin, grp75 and age. This interrelationship suggests that, instead of a single event, the expression of hsp70 and hsp60 is regulated by a combination of factors that involve oxidative stress, calcium and age. The large variation in hsp70 levels between DMD patients (C.V. = 1.07) may reflect differences in anatomical sites chosen for muscle biopsies with regard to the content of hypercontracted fibers, since hsp70 is significantly upregulated in these fibers [5].

Grp75 was significantly increased in DMD, but not altered in DM, PM and MM. This selective upregulation of grp75 in DMD, in comparison to the other myopathies, is most likely related to the elevated intracellular calcium levels found in DMD.

Along with increased grp75 expression in DMD, a significant elevation in GSH Px was evident, suggesting an increased oxidative burden in DMD. Although other oxidation-specific stress proteins such as catalase and HO were prominently upregulated in certain DMD individuals, mean values were not significantly increased. Patients in one experimental group usually have different stages of the disease associated with specific pathological reactions. The heterogeneity of the clinical course of the disease may explain the disparity in reports concerning the oxidant-antioxidant status of DMD patients: indeed, little consensus can be found in this regard and antioxidant therapeutic trials have been predominantly unsuccessful (for review, [9]).

In contrast to the selective upregulation of grp75 in DMD, hsp60 was significantly induced in all myopathies investigated. A common feature of muscle disorders which may account for this uniform induction of hsp60, is altered muscle cell metabolism, insufficient energy production, ATP depletion in itself, or the associated oxidative stress.

Comparison of mean values showed that the increased expression of ferritin was significant only in DM. However this analysis concealed the prominent elevations in ferritin in certain individuals with DMD, PM and MM. Indeed multiple regression analysis revealed that ferritin influences variation in hsp70 and hsp60 levels in DMD. Ferritin is induced by the cytokines, tumor necrosis factor-α (TNFα) and interleukin-Iα 1 (IL-1) [18,19] in skeletal muscle cells. These inflammatory mediators may influence the levels of ferritin. Abundant ferritin has been described in degenerating fibers and in filtrated macrophages in muscle of rabbits during vitamin E-deficiency, which is known to cause an extensive necrotizing myopathy [6]. Ferritin levels in myopathies are regulated by the release of iron from myoglobin/heme by oxidative injury or HO activity within myofibers or infiltrated macrophages. Iron sequestration by ferritin in macrophages [12] and myofibers might serve the beneficial function of lessening the exposure to potentially cytotoxic iron-catalysed oxidants such as $^{\bullet}OH$.

Since age influences both stress responsiveness and basal free radical production, it was included as an independent variable in multiple regression analysis. Concerning DMD, multiple regression analys is revealed a significant interdependence ($p = 0.03$) between hsp70, catalase, GSH Px, ferritin, grp75 and age. The coefficient of determination ($R^2 = 0.99$) indicated that almost 99% of fluctuations in hsp70 levels are explained by alterations in the levels of catalase, GSH Px, ferritin and age. According to multiple regression analysis 99% of variation in hsp60 in DMD is defined by variation in HO, GSH Px, ferritin, grp75 and age. The *p* value (0.14) for this interrelationship was however insignificant.

The most prominent contribution to variation in hsp70 and hsp60 levels as defined by the multiple regression functions (Fig1) was age. Adult muscle is more susceptible to free radical injury than immature muscle [20]. With regard to DMD, age has a large influence on the pathology and clinical manifestations of the disorder. For example, Bonsett and Rudman [2] suggested that calcium-related necrosis represents the disease mechanism most active between 4-5 years of age, a period during which serum CK levels peak. Another age-related change is the decrease in serum carbonic anhydrase activity, conforming to the relation between serum CK and age/severity [13]. In addition, accelerated aging in dystrophic muscle has previously been proposed as a result of an age-related decline in glucocorticoid myogenic stimulation observed in DMD muscle cultures, compared to the enhanced myogenesis in age-matched normal cultures, an aspect that may also reduce *in vivo* myofiber regeneration. Recurrent cycles of degeneration and regeneration accelerate age-related phenomena in DMD. Such an accelerated aging process may account for the large negative effect of age on HSP levels in DMD

muscle and may, at least in part, explain the progressive nature of the disorder.

A proposed model for stress protein induction in DMD has been recently published [3]. In addition, the data presented here provide the first evidence of a strong correlation between hsp70, hsp60, grp75, oxidation-specific stress proteins, ferritin and age in DMD, suggesting that free radical reactions potentiated by calcium and influenced by age relate to muscle degeneration and the activation of stress-related genes. Both HSP and GRP might be involved in muscle protection, and in particular, in protection from apoptosis, in an age-dependent fashion.

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