Heat Shock Does not Affect the Phenotypic Expression of DMPK in Muscle Cells

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Abstract

Myotonic dystrophy is a multisystem disorder associated to a trinucleotide repeat expansion in the 3'-untranslated region of a gene encoding a protein kinase, DMPK. The physiological role of the DMPK is largely unknown. DMPK and other closely related protein kinases interact with members of the Rho family of small GTPases suggesting that they may be involved in signal transduction. On the other hand, the discovered interaction between DMPK and the small heat shock protein MKBP suggests that DMPK might be involved in the response against external stimuli. The possibility exists that DMPK might act as a switch between different intracellular pathways, depending on distinct external signals. We report here that heat shock does not appear to change phenotypic expression of DMPK neither in the whole tissue nor in cultured cells.

Key words: heat shock, muscle, myotonic dystrophy protein kinase, stress proteins, triplet repeat diseases.

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Dystrophia myotonica (DM) was initially described as myotonia atrophica by Steinert [17] and Batten and Gibb [2]. This myotonic muscular dystrophy is a diffuse systemic disorder in which the most prominent features, i.e. myotonia and muscular atrophy, may be accompanied by cataracts, gonadal atrophy, endocrine abnormalities, heart conduction defects, mental deficit. It is inherited as an autosomal dominant trait with a variable penetrance [7].

The molecular basis of DM is the expansion of a CTG repeat in the 3' untranslated region of a gene encoding a protein kinase (DMPK) and located on chromosome 19q13.3 [6-5, 8]. Until recently, DM was considered as a single nosological entity. However, recent findings indicate that a milder form of DM, formerly known as proximal myotonic myopathy (PROMM), is distinct from the more severe form, the molecular lesion being a CCTG expansion on chromosome 3q21 [11, 14]. As a consequence, the two conditions have received a new designation: DM1 and DM2, respectively. To explain how this kind of mutation, which does not alter the protein-coding portion of the gene, may cause the large spectrum of symptoms of DM1, three possible pathogenetic mechanisms have been proposed: a) a decrease of the DMPK expression (haploinsufficiency), probably responsible for cardiac defects; b) an altered expression of the flanking genes SIX5 and DMWD, possibly involved in cataract and cognitive defects, respectively; c) a toxic gain of function, due to the accumulation of CUG-binding proteins and, consequently, to the misregulation of the RNA metabolism. Decreased expression level of DMPK has been actually reported in both DM skeletal muscles [20] and cells [1].

Little is known about the physiological properties of DMPK. It belongs to a group of protein kinases that interact with members of the Rho family of small GTPases that appear to control cell size and shape in a variety of organisms [16]. Several localization sites have been reported for DMPK, including neuromuscular and myotendinous junctions [22] and cytoplasmic surface of SR, suggesting that it may be involved in the regulation of excitation-contraction coupling of skeletal muscle [15, 20]. In addition, in cardiac muscle DMPK was demonstrated at the cytoplasmic face of both junctional and extended junctional SR, as well as associated to connexin 43, at the gap junctions of the intercalated discs, suggesting it may also play a modulative role in the transmission of signals between myocytes [12].

Other experimental data suggest that the altered expression of DMPK in DM muscles may lead to an increased susceptibility toward physico-chemical stimuli [21]. Interestingly, Suzuki et al. [19] reported that DMPK associates specifically with a new member of the small heat shock proteins (sHSP), accordingly named myotonic dystrophy protein kinase binding protein (MKBP).

The stress proteins may have a double function, by exerting a chaperone-like activity or a cytoprotective role. To follow the muscle response to different stress conditions we have initially chosen heat shock and oxidative stress. In this report we show preliminary results on the effect of temperature on the expression of sHSPs and DMPK in both myogenic cultured cells and freshly excised muscle tissue.

Materials and Methods

Male albino Wistar rats (200 g body weight) were used as a source of skeletal (soleus) muscles. Soleus muscle freshly excised from rat hind legs was immediately incubated at 37°C or 44°C for 20 min, essentially as described by Kato et al. [9]. After treatment, muscles were rapidly frozen in liquid nitrogen, ground into powder and then homogenized in 50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT in the presence of a cocktail of protease inhibitors. The homogenate was centrifuged at 100,000 g for 90 min to obtain cytoplasmic and membrane fractions.

C2C12 mouse myoblasts were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Differentiation was induced switching the cells to DMEM supplemented with 2% horse serum. After 4 days, heat shock was induced by floating the flasks in water at 44°C for 15 min, essentially as described by Sugiyama et al. [18]. The cells were then recovered for 1 to 8 hours at 37°C and finally harvested and lysed in 5% deoxycholate containing a cocktail of protease inhibitors.

Anti-DMPK antibody was prepared as previously reported [15]. Anti-HSP70, anti-αB-crystallin, anti-HSP25 antibodies were from Stressgen.

SDS-PAGE electrophoresis was carried out as described by Laemmli [10]. Immunoblotting was carried out as previously reported [15]. Immunoblots were developed with the ECL system (Amersham) or, alternatively, with NBT/BCIP system (Pierce).

Results and Discussion

Muscle cells are usually subjected to different stimuli as mechanical, heat, metabolic and oxidative stresses. Changes in the expression of HSPs have been reported for some myopathies as for instance Duchenne muscular dystrophy [3]. To see if heat shock conditions may alter DMPK expression we used both cultured cells and whole tissue.

First, C2C12 mouse myotubes after four days of differentiation were subjected to heat shock essentially as described by Sugiyama et al. [18] at 44°C for 15 min. After a recovery period ranging from 1 to 8 hours at 37°C, cells were harvested and lysed. Fig. 1 shows that the synthesis of HSP25 and of the inducible HSP70 were activated after a recovery period of 2-4 hours. On the contrary, DMPK did not show any change when the same samples were reacted with TA1, an anti-DMPK antibody directed against a C-terminal epitope.

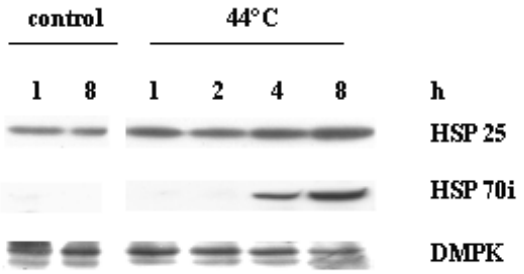

Figure 1. Western blot analysis of C2C12 myotubes after heat shock. C2C12 myotubes were heat treated at 44°C for 15 min and then recovered at 37°C for the indicated times. Equal amounts of cell lysate were loaded per well (20 μg for HSP25, 50 μg for HSP70 and DMPK). The blots were developed by using antibodies against the indicated proteins.

Second, soleus muscle freshly excised from rat hind legs was immediately incubated at 46°C for 20 min, essentially as described by Suzuki et al. [19]. Muscle homogenate, resuspended pellet and supernatant were analyzed by immunoblotting. Fig. 2 shows that heat treatment induced an increase of αB-crystallin and HSP25 synthesis together with their translocation from the supernatant to the pellet. At variance, DMPK as detected by TA1 did not show any changes in its level. The expression of the inducible form of HSP70 was similarly not affected (data not shown), probably due to the lack of recovery after treatment. Taken together these results seem to indicate that temperature-induced stress does not influence the phenotypic expression of DMPK neither in normal skeletal muscle nor in the C2C12 myogenic cell line.

Suzuki et al. [19] showed that MKBP is not induced by heat shock. Besides, MKBP seems to activate recombinant DMPK autophosphorylation and to protect the enzyme from heat-induced inactivation more effec-

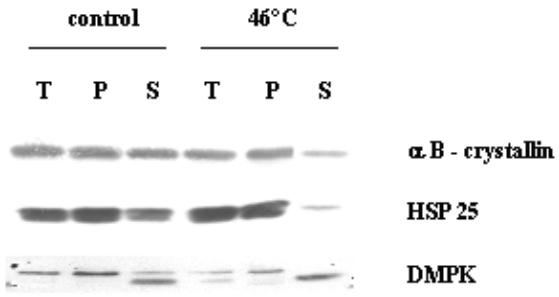

Figure 2. Western blot analysis of fractions isolated from muscle tissue after heat treatment. Rat soleus muscle was treated at 46°C for 20 min and then processed as described in the Materials and Methods section. Control was incubated at 37°C for the same time. Equal amounts of proteins from total homogenate (T), isolated membrane pellet (P) and soluble cytosolic fraction (S) were loaded per well (10 μg for αB-crystallin, 50 μg for HSP25 and DMPK). The blots were developed by using antibodies against the indicated proteins.

tively than other sHSPs. MKBP up-regulation in DM muscles, interpreted as a feed-back response to the DMPK haploinsufficiency has been also reported [19]. Likely, the presence of unstable triplet repeat in the 3' untranslated region of the DMPK gene may be responsible for the increased susceptibility to injury, as proposed by Usuki and Ishiura [21] for the oxidative stress.

Future Directions

Muscle cells usually undergo mechanical, heat, metabolic and oxidative stresses. The stress-responsive system of muscle cells may be regulated depending on the type of stimuli. Investigating the response to the oxidative stress in slow-twitch skeletal muscles may be a major point, since type I (slow) muscle fibers which are characterized by an oxidative metabolism, undergo a selective atrophy in DM patients. Moreover, slow muscles have the higher content of HSPs.

It is well known that oxidative stress can induce apoptosis. Thus, experimental conditions have to be chosen to avoid muscle cells may enter the apoptotic pathway. This consideration arises from the work by Suzuki et al. [19] who suggests that DMPK and MKBP synthesis are inversely correlated and from the observations of Yamada et al. [23] who detected DNA fragmentation in DM patients, suggesting a significant role of apoptosis in the pathogenesis of the disease. It remains to establish if apoptosis and stable up-regulation of MKBP are linked each other. On the other hand, if DMPK expression is somehow correlated to HSP synthesis, it would be useful to modify muscle cells by decreasing their DMPK content by using an anti-sense RNA, and to follow the HSP expression under the previously assessed oxidative conditions. Alternatively, the DMPK expression might be increased by inserting several copies of the corresponding cDNA. In this case, the possibility that a sHSP might form a complex with DMPK would be easily followed by immunoprecipitation or binding studies.

Moreover, taking into account that the suggested dysfunction of RNA metabolism in DM cells may have a secondary effect on the synthesis of proteins responsible for either cell cycle regulation or calcium homeostasis, muscle cells up- and down-regulated for DMPK expression might be also effective in verifying the susceptibility to oxidative stress during differentiation. Since overexpression of DMPK in C2C12 cells has been reported to cause a decreased level of fusion to the terminally differentiated state [13], it would be interesting to correlate this delay of maturation with the stress-response capability.

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