Expression of stress proteins and mitochondrial chaperonins in chronically stimulated skeletal muscle

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Molecular chaperones and cytosolic stress proteins are actively involved in the stabilization, import and refolding of precursor proteins into mitochondria. The purpose of the present study was to evaluate the relationship between mitochondrial content under steady-state conditions, and during the induction of organelle biogenesis, with the expression of stress proteins and mitochondrial chaperonins. A comparison of steady-state levels of mitochondrial enzyme activity [cytochrome *c* oxidase (CYTOX)] with chaperonin levels [the heat-shock protein HSP60, the glucose-regulated protein GRP75 (mtHSP70)] in striated muscles possessing a wide range of oxidative capacities revealed a proportional expression between the two. This relationship was disrupted by chronic contractile activity brought about by 10 days of 10 Hz stimulation of the tibialis anterior (TA) muscle, which induced 2.4-fold increases in CYTOX activity, but 3.2- and 9.3-fold increases in HSP60 and GRP75 respectively. The inducible stress protein $HSP70_i$ was detected at low levels in control TA muscle, and was increased 9.6-fold by chronic contractile activity, to values comparable with those found in the unstressed soleus muscle. This increase occurred in the absence of changes in type I MHC levels, indicating independent regulation of these genes. Despite the increases in HSP60 and $HSP70_i$ proteins, contractile activity did not alter their respective mRNA levels, illustrating post-transcriptional mechanisms of gene regulation during contractile activity. In contrast, the mRNA levels encoding the co-chaperonin CPN10 were increased 3.3-fold by contractile activity. Thus, the expression of individual mitochondrial chaperonins is independently regulated and uncoordinated. The extent of the induction of these stress proteins and chaperonins by contractile activity exceeded that of membrane enzymes (e.g. CYTOX). It remains to be determined whether this marked induction of proteins comprising part of the protein import machinery is beneficial for the translocation of enzyme precursors into the mitochondria during conditions of accelerated biogenesis.

INTRODUCTION

Skeletal muscle is a tissue which can undergo multiple phenotypic adaptations in response to physiological demands. One physiological stress which is commonly used to study the adaptability of muscle is that of chronic contractile activity induced by low-frequency (10 Hz) stimulation, which dramatically alters muscle biochemical and functional properties [1–3]. Among the multitude of alterations that occur, chronic stimulation induces mitochondrial biogenesis, and converts the muscle from white to red in its phenotypic appearance [1–9]. These adaptations represent dynamic transitions in muscle fibre composition. It is also well known that, under steady-state conditions, rat hindlimb muscles exist as collections of fibre types which are predominantly composed of type I, IIa or IIb myosin heavy chain (MHC), possessing wide variations in mitochondrial content. A comparison of the pattern of gene expression under steady-state conditions, with that under conditions of adaptation such as that induced by chronic stimulation, can provide insight into the plasticity of skeletal muscle. Of interest to us is the expression of stress proteins and mitochondrial chaperonins in this tissue. Stress proteins are involved in the folding, stabilization and translocation of nascent polypeptides into cellular organelles [10,11]. For example, it has been shown that cytoplasmic stress proteins of the HSP70 family of heat-shock proteins interact transiently with newly synthesized polypeptides on polysomes and stabilize unfolded proteins in a translocation-competent form, thus facilitating the entry of proteins into mitochondria [12,13]. Two constitutive stress proteins are also known to be present within mammalian mitochondria, and these fulfil essential roles as 'chaperonins' [14,15]. Because of their functions and apparent size when subjected to SDS/PAGE, the proteins are referred to as the 60 kDa heat-shock protein (HSP60) and the 75 kDa glucose-regulated protein (GRP75), also known as the mitochondrial HSP70 [12]. As the unfolded protein stabilized by cytoplasmic HSP70s enters the mitochondria, it interacts with GRP75, which drives the translocation of the precursor across the membrane by binding to unfolded segments appearing on the matrix side [16]. The precursor is then released in an ATP-dependent process, and is bound to the the 14-subunit HSP60 complex, which acts as a scaffold for protein refolding and oligomer assembly [13,17]. Mitochondria also contain a co-chaperonin referred to as chaperonin 10 (CPN10 [18,19]). This 10 kDa protein appears to assist the function of HSP60, and it is an essential component of the protein folding apparatus, required for assembly and sorting functions [20]. At this time, nothing is known regarding the expression of chaperonins in skeletal-muscle fibre types, and during the process of adaptation.

Recently, a number of studies have begun to document the expression of HSPs in specific skeletal muscles [21] and as a result of exercise [22–24]. Particularly emphasized in these studies was the protein level of expression of the inducible HSP70, denoted $HSP70_i$. It has been shown that rat muscle fibres containing type II MHC do not express $HSP70_i$, whereas fibres rich in type I

Abbreviations used: HSP, heat-shock protein; GRP, glucose-regulated protein; CPN, chaperonin; MHC, myosin heavy chain; mtHSP70, mitochondrial heat-shock protein; CYTOX, cytochrome *c* oxidase; TA, tibialis anterior; FTR, fast-twitch red; FTW, fast-twitch white; STR, slow-twitch red; HSF, heat-shock factor.

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MHC have greater constitutive amounts of this protein [21]. When the rat tibialis anterior (TA) muscle is chronically stimulated at 10 Hz, decreased amounts of type IIb MHC occur, along with concomitant increases in type IIa MHC [25], and 2-fold increases in mitochondrial content between 5 and 10 days [26]. These dramatic alterations in muscle phenotype resulting from changes in cellular protein turnover might represent a stimulus for the enhanced expression of stress proteins [9], especially those connected with protein import and folding to support the coincident increase in mitochondrial biogenesis. Thus, the purpose of this study was to examine the $HSP70_i$, HSP60, CPN10 and GRP75 expression in relation to mitochondrial content under steady-state conditions, and relative to mitochondrial biogenesis under conditions of chronic contractile activity.

MATERIALS AND METHODS

Antibodies and cDNAs

All materials purchased were of the highest chemical grade. Monoclonal antibodies against the stress proteins HSP60, GRP75 and $HSP70_i$, as well as cDNAs encoding HSP60 and $HSP70_i$, were obtained from StressGen Biotechnologies, Victoria, BC, Canada. A monoclonal antibody specific for type I MHC was provided by Dr. P. A. Merrifield, University of Western Ontario, Canada. The cDNA encoding CPN10 from rat liver was provided by Dr. Peter B. Høj, La Trobe University, Australia.

Animals and stimulation

Adult male Sprague–Dawley rats were anaesthetized with sodium pentobarbitol (65 mg/kg), and surgical electrodes were implanted unilaterally on both sides of the common peroneal nerve of the left hindlimb, as done previously [7,26]. After a 7-day recovery period, the TA muscle was chronically stimulated at 10 Hz (24 h/day) for 10 days ($n = 10$). The contralateral right TA muscle was used as the non-stimulated control. After stimulation, the animals were killed and muscle samples were excised, snap-frozen and powdered under liquid N_2. A series of normal animals ($n = 4$) was also used for the removal of specific tissues. Heart, liver, red and white gastrocnemius and soleus muscles were removed and frozen as described above.

Muscle homogenates and extracts

Homogenates from powdered tissues were prepared in 100 mM Na/K phosphate/2 mM EDTA buffer (pH 7.2) by sonication (8 × 10 s) on ice [7,27]. The homogenates were used to determine cytochrome *c* oxidase (CYTOX; EC 1.9.3.1) activity [27] and total protein concentration [28]. Muscle extracts were similarly prepared, except that the myofibrillar fraction was removed by centrifugation in a Microfuge at 4 °C. Extracts were used to measure the concentration of stress proteins by immunoblotting.

Immunoblotting

One-dimensional SDS/PAGE of muscle samples (150–500 μg of protein/lane) was performed in 10% gels, followed by immunoblotting [29]. After protein electrotransfer, the nitrocellulose membranes were blocked with 5% non-fat milk/PBS, and incubated for 2 h with anti-HSP60, anti-GRP75 or anti-$HSP70_i$ antibodies at working dilutions of 1:800, 1:1000 and 1:500 respectively. Sheep anti-mouse IgG conjugated to alkaline phosphatase was used as a secondary antibody. Alkaline phosphatase binding was revealed by a colour reaction which developed within 5 min, and signals were quantified by laser densitometry.

Total RNA isolation and hybridization

Total RNA was isolated from frozen muscle powders as described previously [30]. The quality of each mRNA sample was assessed by electrophoresis in formaldehyde/1%-agarose gels. RNA samples (10 μg) were transferred to nylon membranes using a vacuum manifold slot-blot apparatus. Slot-blots were pre-hybridized for 3 h and hybridized overnight with a ^{32}P-labelled cDNA probe encoding HSP60, CPN10 or $HSP70_i$ at 42 °C, as done previously [7]. The blots were stringently washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% SDS at 60 °C for 30 min (HSP60, CPN10 cDNAs) or 15 min ($HSP70_i$ cDNA) and exposed to autoradiography film at −80 °C. Signals were quantified by laser densitometry.

Statistics

Statistical analysis was done by Student's paired *t* test. The data are expressed as means ± S.E.M., and values of $P < 0.05$ were used to indicate statistical significance.

RESULTS

Relationship of HSP60 and GRP75 expression to enzyme activity in striated muscles

The presence of HSP60 and GRP75 was demonstrated by immunoblot analysis in rat liver and striated muscles (Figure 1, inset). Liver samples were included only for comparative purposes in immunoblots, but these were not quantified. Densitometric scanning of multiple immunoblots showed that heart possessed the highest content of HSP60, followed by the fast-twitch red (FTR) fibres of the red gastrocnemius muscle, the slow-twitch red (STR) fibres of the soleus, and the fast-twitch white (FTW) fibres of the white gastrocnemius muscle (Figure 1). A very similar pattern of expression was found with GRP75. CYTOX activity was determined in all muscle samples and used as a

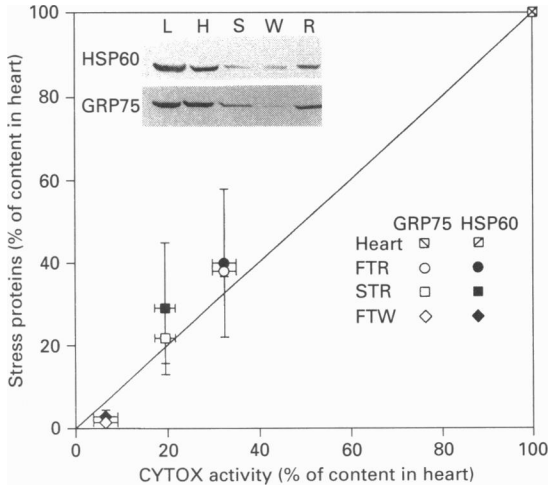

Figure 1 Proportional expression of mitochondrial HSP60 and GRP75 with CYTOX in rat striated muscles

Mitochondrial HSP60 and GRP75 were detected by immunoblotting (150 μg of total protein/lane, inset). Liver (L) and heart (H) possess the highest content of HSP60 and GRP75, followed by the red gastrocnemius (R), soleus (S) and white gastrocnemius (W). Relative amounts of HSP60 and GRP75 are shown as a percentage of the protein level detected in the heart, and compared with CYTOX values, also expressed as a percentage of values found in heart. The line of identity is drawn to facilitate comparisons of 1:1 relationships.

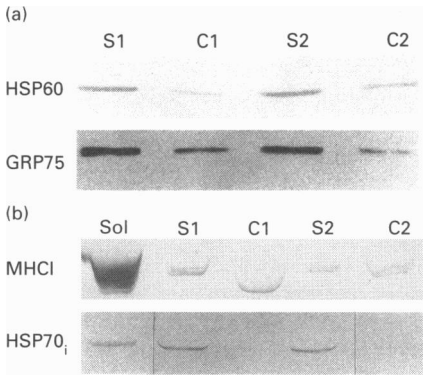

Figure 2 Protein levels in chronically stimulated, control and soleus muscles

(**a**) Immunoblots of HSP60 and GRP75 proteins in 10-days-stimulated (S1, S2) TA and contralateral non-stimulated muscle (C1, C2) extracts. Gels were loaded with 500 μg of total protein/lane. (**b**) Immunoblots of HSP70$_i$ and type I MHC proteins in soleus muscle (Sol), stimulated (S1, S2) TA and contralateral non-stimulated TA muscle (C1, C2) extracts. Separate gels were loaded with 500 μg of total protein/lane for HSP70$_i$ detection, or 50 μg of total protein/lane for type I MHC detection.

marker of mitochondrial content [31]. Heart possessed the highest mitochondrial content (54.1 $\pm$ 4.4 units/g wet wt.; $n = 7$), whereas the content in FTR, STR and FTW was 32.6%, 19.6% and 6.5% of that in heart, respectively. Figure 1 illustrates the relationship between tissue CYTOX activity and HSP60 and GRP75 levels. It is evident that the expression of mitochondrial HSP60 and GRP75 parallels the content of mitochondria under normal steady-state conditions in the heart and different skeletal-muscle types.

Expression of mitochondrial HSP60 and GRP75 in chronically stimulated muscle

Chronic contractile activity for 10 days induced mitochondrial biogenesis, as evident from the 2.4 $\pm$ 0.3-fold ($n = 7$) increase in CYTOX activity measured in homogenates of stimulated TA muscle (20.0 $\pm$ 1.9 units/g wet wt.) compared with the contralateral muscle (7.9 $\pm$ 1.8 units/g wet wt.). HSP60 and GRP75 were quantified in the same samples. Immunoblots (Figure 2a) indicated that HSP60 levels were 3.2 $\pm$ 0.9-fold higher ($n = 8$), and GRP75 levels were 9.3 $\pm$ 2.1-fold higher ($n = 8$), in stimulated compared with non-stimulated TA muscle. In addition, the increase in GRP75 due to contractile activity exceeded ($P < 0.05$) that of HSP60 by approx. 3-fold.

Expression of HSP70$_i$ in chronically stimulated muscle

HSP70$_i$ is normally almost undetectable in control TA muscle containing a large fraction of type II MHC, but this protein was found in abundance in the type I MHC-rich soleus muscle [21]. Values in soleus muscle were 10.6 $\pm$ 1.5-fold higher ($n = 8$) than in the non-stimulated control TA (Figure 2b). Chronic contractile activity had a profound effect on the expression of HSP70$_i$ in the TA muscle. The relative increase observed was 9.6 $\pm$ 0.6-fold higher than in control muscle ($n = 8$), attaining levels similar to those found in the soleus. However, there was no coincident change in the amount of type I MHC in stimulated TA, compared with control TA muscle (Figure 2b).

Table 1 Relative amounts of specific mRNA levels in striated muscles

Values are means $\pm$ S.E.M. ($n = 3$–10). The data represent ratios of levels in stimulated TA (sTA) to those in the respective contralateral non-stimulated muscle (cTA). Also shown are ratios of levels in normal soleus muscle (Sol) and heart (Ht) to levels in non-stimulated TA. * $P <$ 0.05 for stimulated TA versus control.

Specific mRNA ratio	HSP60	CPN10	HSP70$_i$
sTA/cTA	1.38 $\pm$ 0.18 (7)	3.32* $\pm$ 0.48 (10)	1.23 $\pm$ 0.15 (9)
Sol/cTA	1.16 $\pm$ 0.03 (5)	1.08 $\pm$ 0.08 (6)	1.07 $\pm$ 0.04 (6)
Ht/cTA	1.45 $\pm$ 0.11 (3)	0.62 $\pm$ 0.12 (3)	0.94 $\pm$ 0.12 (3)

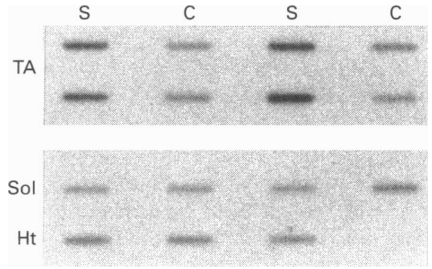

Figure 3 CPN10 mRNA levels in striated muscles

Slot-blot analysis of CPN10 mRNA in chronically stimulated (S) and contralateral non-stimulated TA muscle (C), as well as normal soleus (Sol) and heart (Ht) muscles; 10 μg of total RNA was loaded per slot.

HSP60, CPN10 and HSP70$_i$ mRNA levels in chronically stimulated muscle

Chronic contractile activity did not induce significant increases in HSP60 or HSP70$_i$ mRNA levels (Table 1) in the TA muscle. Ratios of stimulated/control mRNA levels did not differ significantly from 1. However, the levels of CPN10 mRNA were 3.3-fold higher in the stimulated TA compared with control muscle ($P < 0.05$), whereas control CPN10 mRNA levels did not differ from those observed in soleus or heart (Figure 3, Table 1).

DISCUSSION

Contractile activity is a physiological stress in which the synthesis and degradation of proteins directly involved in muscle contraction and energy provision are stimulated [1,2]. In particular, it is a condition which stimulates mitochondrial biogenesis in muscle (for reviews see [1,8,9]). We hypothesized that contractile activity would trigger the synthesis of stress-inducible proteins and chaperonins which are involved in organelle biogenesis. As outlined in the Introduction, cytosolic proteins of the HSP70 family, as well as matrix chaperonins (HSP60, GRP75, CPN10), play a fundamental role in the import, folding and localization of nuclear gene products which are essential for mitochondrial structure, function and biogenesis. We evaluated the expression of these proteins in striated muscle types which differ vastly in their steady-state mitochondrial contents, and during contractile-activity-induced augmentations in mitochondrial biogenesis. Under steady-state conditions, our results indicate that the tissue content of the mitochondrial chaperonins GRP75 (mtHSP70) and HSP60 is proportional to muscle oxidative capacity and

mitochondrial content, as represented by CYTOX activity [31]. However, this proportionality (Figure 1) is altered by chronic contractile activity, such that the one-to-one relationship is no longer maintained. Chronic stimulation typically results in 2–3-fold increases in mitochondrial enzyme activity in rat fast-twitch muscle [7,26]. It is evident from our data, and from the data of others (see [9] for review), that not all mitochondrial proteins are increased to the same extent by contractile activity. In the present study, an apparent overproduction (by 1.3–3.9-fold) of GRP75 and HSP60 was observed, exceeding the increase in a typical inner-membrane enzyme (CYTOX). In addition, the increases in these two stress proteins differed markedly from each other. This indicates that the expression of these chaperonins is differentially regulated by contractile activity. We hypothesize that they may be over-induced (relative to enzyme induction), not only to keep pace with mitochondrial biogenesis, but to ensure that the rate of protein import into the growing mitochondria of stimulated muscle is not a rate-limiting process in organelle synthesis. In support of this, we have recently found that, during hypothyroidism-induced decreases in mitochondrial enzyme activity, HSP60 and GRP75 are nonetheless maintained at normal levels [32].

The synthesis of HSP60 and $HSP70_i$ appear to represent interesting examples of post-transcriptional regulation. This is inferred from the lack of increase seen in the levels of mRNA encoding these two proteins, at the same time as protein measurements indicated 3–9-fold increases as a result of 10 days of chronic contractile activity. One interpretation of these results is that an increased stability of these proteins exists relative to their respective mRNAs, possibly accompanied by the transient induction of the genes encoding HSP60 and $HSP70_i$ at the onset of the contractile-activity stimulus. Indeed, Hand et al. [33] have reported temporary increases in HSP60 and $HSP70_i$ mRNA levels during the first 3 days of chronic stimulation, followed by subsiding levels thereafter in rabbit muscle. Alternatively, it has been shown in cells responding to heat shock that HSP70 mRNA translation occurs preferentially relative to other cellular mRNAs [34]. A post-transcriptional mechanism of this type might account for large increases in protein level in the absence of marked changes in mRNA level. In contrast with the lack of change in HSP60 and $HSP70_i$ mRNAs, levels of mRNA encoding CPN10 were significantly increased by more than 3-fold in stimulated muscle. Thus, although HSP60 and CPN10 function in concert during protein folding in the mitochondria [20], our data suggest that the expression of these two chaperonin genes is differentially regulated.

It has been demonstrated that the constitutive expression of $HSP70_i$ is roughly proportional to the type I muscle fibre composition [21]. Our data indicate that this relationship is not maintained during physiological stress imposed by chronic contractile activity. After 10 days of chronic stimulation, the trace amounts of type I MHC in the TA muscle remained unchanged (Figure 2), whereas the amount of type IIb/IIx MHC decreased ([25]; O. I. Ornatsky and D. A. Hood, unpublished work). Despite this, a 9-fold increase in the level of $HSP70_i$ was evident. Thus, MHC and $HSP70_i$ gene expression are independently regulated in skeletal muscle, similarly to recent results documented in heart [35]. It is well known that the expression of the HSP70 family of genes is controlled by heat-shock factors (HSF1, HSF2, HSF3), which interact with upstream regulatory regions containing specific heat-shock-inducible elements [36]. Future work examining the function of these HSFs in skeletal muscle may help to explain the augmented expression of $HSP70_i$ resulting from chronic stimulation. In addition, the tissue distribution of these HSFs [37] could account for the tissue-specific expression of $HSP70_i$. In other studies we (O. I. Ornatsky and D. A. Hood, unpublished work) and others [35] have failed to detect the presence of $HSP70_i$ in the normal rat heart. Despite this, mRNA levels encoding $HSP70_i$ in the heart are similar to those found in the soleus (Table 1), a tissue which expresses the protein at a high level (Figure 2). This suggests that the control of $HSP70_i$ levels in various tissues such as the heart is post-transcriptional, possibly at the level of translation or protein stability.

HSP60 is known to be essential for cell viability in yeast [14], and deficiency of the protein appears to be clinically relevant, implicated as the cause of a mitochondrial disorder [38]. In addition, HSP60 mRNA is decreased in conditions of copper deficiency [39], and augmented in hepatoma cells supplemented with cadmium [40]. Apart from this, and in contrast with the wealth of emerging literature on the HSP70 genes [36], nothing appears to be known regarding the regulation of the expression of the mitochondrial chaperonins HSP60, GRP75 or CPN10. Chronic low-frequency stimulation of rat skeletal muscle appears to be a useful model for investigating the relationship between mitochondria biogenesis, gene expression and the signals responsible for the induction of stress proteins and mitochondrial chaperonins.

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