Expression of endoplasmic reticulum stress proteins during skeletal muscle disuse atrophy

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Expression of endoplasmic reticulum stress proteins during skeletal muscle disuse atrophy. Am J Physiol Cell Physiol 281: C1285–C1290, 2001.—Disuse atrophy of skeletal muscle leads to an upregulation of genes encoding sarcoplasmic reticulum (SR) calcium-handling proteins. Because many of the proteins that are induced with endoplasmic reticulum (ER) stress are ER calcium-handling proteins, we sought to determine whether soleus muscle atrophy was associated with a prototypical ER stress response. Seven days of rat hindlimb unloading did not alter expression of ubiquitous ER stress proteins such as Grp78, calreticulin, and CHOP/GADD-153, nor other proteins that have been shown to be activated by ER stressors such as vimentin, the type I d-myo-inositol 1,4,5-trisphosphate receptor, or protein kinase R, a eukaryotic initiation factor 2α kinase. On the other hand, expression of heme oxygenase-1 (HO-1), an antioxidant ER stress protein, was significantly increased 2.2-fold. In addition, unloading led to an increase in calsequestrin, the muscle-specific SR calcium-binding protein, at both the mRNA (68%) and protein (24%) levels. Although disuse atrophy is associated with a significant remodeling of muscle-specific proteins controlling SR calcium flux, it is not characterized by a prototypical ER stress response. However, the upregulation of HO-1 may indicate ER adaptation to oxidative stress during muscle unloading.

unloading; sarcoplasmic reticulum; heme oxygenase; CHOP/GADD-153; vinculin; 78-kDa glucose-regulated protein; calreticulin; calsequestrin; calcium; inositol trisphosphate receptor; protein kinase R

AN UNEXPECTED ASPECT OF MUSCLE remodeling associated with disuse atrophy is the marked induction of proteins that regulate intracellular calcium flux from the primary intracellular storage site, the sarcoplasmic reticulum (SR). This remodeling is observed as early as 1 wk after removal of weight bearing in rat soleus muscles, and it includes the ryanodine receptor (15), the dihydropyridine receptor (14, 15), and the sarcoplasmic reticulum Ca2+-ATPase (SERCA1) (24, 28). These observations suggest a modified capacity for intracellular calcium flux. Indeed, the rate of SR calcium uptake and release are faster in unloaded soleus muscles (28, 30), and the amount of passive calcium leakage from the SR is greater in unloaded solei (30).

Consistent with these observations, there is a doubling of free cytosolic calcium concentration in soleus muscle fibers after 7 days of unloading (13).

Interestingly, this remodeling of skeletal muscle in response to muscle disuse appears similar to the universal response of eukaryotic cells to endoplasmic reticulum (ER) stressors. These stressors include ER calcium depletion, unfolded protein accumulation, and glucose deprivation (16). The prototypical ER stress response leads to induction of proteins involved in regulating ER calcium flux, and the genes encoding these proteins contain a conserved sequence (CCAATnCCACG) in their regulatory region called the ER stress-response element. Many of the ER stress-response genes encode ER calcium-binding proteins that also act as chaperones during unfolded protein accumulation. Besides ER calcium-binding proteins, the ubiquitous ER Ca2+-ATPase gene (SERCA2b) has recently been shown to be a bona fide ER stress protein (8). Transactivation of these genes by ER stressors is required for survival and adaptation to stress (16). ER stressors also lead to the activation of the transcription factor CHOP/GADD-153 (CCAAT/enhancer-binding protein homologous protein/growth arrest and DNA damage inducible) and to the phosphorylation of the eukaryotic initiation factor 2α (eIF-2α) (16). Phosphorylation of eIF-2α by “stress kinases” such as protein kinase R (PKR) leads to the inhibition of protein translation (16). If an ER stress stimulus is sustained or severe, the activation of CHOP and eIF-2α leads to apoptosis (16). An important aspect of ER remodeling in response to ER stressors is the normalization of intracellular calcium homeostasis, which would otherwise lead to cell death.

Another ER stress protein is exemplified by the antioxidant protein heme oxygenase-1 (HO-1). HO-1 is localized to microsomes, and it can be activated by oxidative stress in muscle cells (5). It is well known that hypoxia (21) and agents that cause oxidative stress lead to activation of HO-1 in a variety of cell types (1). Treatment of cells with ER calcium-depletion...
agents leads to the activation of HO-1 (22) in addition to the ER stress proteins mentioned above, and it leads to the generation of reactive oxygen intermediates (23).

Because of the similarity in the upregulation of ER genes in response to ER stressors and the upregulation of SR genes in response to muscle atrophy, we explored whether disuse atrophy is associated with a prototypical ER stress response. In the present work, we show that after 7 days of muscle disuse by rat hindlimb unloading, the skeletal muscle-specific SR calcium-binding protein calsequestrin was upregulated, but prototypical ER stress proteins such as calreticulin, Grp78 (78-kDa glucose-regulated protein), CHOP, and the stress kinase PKR, were not upregulated by unloading. Because many ER stressors are associated with the production of reactive oxygen intermediates (23) and upregulation of HO-1 (22), we measured its expression after unloading and found it to be upregulated 2.2-fold. Although muscle disuse atrophy is associated with significant remodeling of the proteins involved in SR calcium flux, the only ER stress protein induced was HO-1, an antioxidant ER stress protein. Because of the upregulation of muscle-specific SR calcium-handling genes and of HO-1, it is possible that muscle disuse atrophy is characterized by a “muscle-specific” SR stress rather than a prototypical ER stress.

METHODS

Hindlimb unloading. Two-month-old female Wistar rats were randomly assigned to control or hindlimb-unloading groups. To induce muscle unloading, the rats’ hindlimbs were suspended by elastic tail casts, as described previously (15). After 1 or 7 days, control and unloaded animals were anesthetized with pentobarbital sodium (40 mg/kg), and soleus muscles from right and left hindlimbs were removed, quickly weighed, and immediately processed for RNA or protein isolation.

Protein isolation procedures. Whole muscle lysates were isolated by homogenizing freshly excised muscle in lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], sonicating 4 × 5 s, shaking for 45 min at 4°C, centrifuging at 15,000 g for 10 min, and using the supernatant as lysate. Microsomal isolations were prepared according to Cameron et al. (7). Briefly, muscles were homogenized in a buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mg/ml leupeptin, 1 mg/ml pepstatin, and 0.1 mg/ml PMSF. The homogenate was centrifuged at 45,000 g for 10 min. The pellet was resuspended in buffer without β-mercaptoethanol, solubilized in 1% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate for 20 min, and centrifuged at 45,000 g for 20 min. The supernatant was used as microsomal protein. Crude muscle membranes were prepared as described by Araki et al. (2). Muscle was homogenized in a buffer containing 1% Triton X-100, 10% glycerol, 1% Nonidet P-40, 50 mM HEPES (pH 7.4), 100 mM sodium pyrophosphosphate, 100 mM sodium fluoride, 10 mM EDTA, 5 mM sodium orthovovanadate, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1.5 μg/ml benamidine, and 0.2 mM PMSF. Homogenates were allowed to solubilize for 1 h at 4°C before centrifuging at 55,000 rpm for 1 h. The supernatants were then used for immunoblotting. Determination of protein concentration was performed by using a detergent-compatible assay (Bio-Rad).

Antibodies. Primary antibodies used for immunoblotting were obtained from Affinity BioReagents (calsequestrin, MA3-913; calreticulin, PA3-900; Grp78, PA1-014; and IP3R-1, PA3-901), Santa Cruz Biotechnology (CHOP/GADD-153, SC-7351), Chemicon (vinculin, MAB1624), Transduction Laboratories (PKR, P97220), and Stressgen (HO-1, SPP-730). Secondary antibodies used for immunoblotting were obtained from Vector Laboratories (horseradish peroxidase-labeled horse anti-mouse) and Transduction Laboratories (horseradish peroxidase-labeled goat anti-rabbit).

Western analysis. Thirty micrograms of protein from soleus or extensor digitorum longus (EDL) muscle samples were denatured in SDS loading buffer, boiled for 3 min, centrifuged briefly to remove insoluble material, and separated on SDS-polyacrylamide gels. Protein was transferred to nitrocellulose and probed with antibodies against calreticulin, Grp78, vinculin, IP3R-1, and IP3R-2. Equal protein loading was confirmed using antibodies specific for vinculin (C). The Western blot images are representative of three to four independent experiments. For measurements of protein concentration, samples were analyzed using a detergent-compatible protein assay reagent (Bio-Rad).
onto a Hybond Enhanced Chemiluminescence (Amersham) nitrocellulose membrane. Membranes were blocked in 5% nonfat milk diluted in Tris-buffered saline-Tween for 1 h and then incubated for 1–2 h with the appropriate antibody, diluted according to the manufacturer’s instructions. Horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence detection system (Amersham) was used for visualization. Extracts used for positive controls were obtained from the following manufacturers: serum-starved 3T3 whole cell lysates, Santa Cruz; recombinant rat HO-1, Stressgen; and PC-12 cell lysates, Transduction Laboratories. Cerebellar microsomal extracts were isolated from rat brain for positive and negative controls where indicated.

**RNA isolation and Northern blotting.** Right and left soleus muscles were combined and homogenized in denaturing solution, and RNA was isolated using the guanidinium-thiocyanate method (9). Total RNA was size fractionated and transferred to nylon membranes using standard procedures (3). Membranes were prehybridized in Ultrahyb (Ambion) for 1 h at 42°C. Random primed cDNA probes using 32P incorporation were then added for overnight incubation. Blots were washed at high stringency and exposed to X-ray film for autoradiography. For the calcequestrin probe, a rabbit cDNA (gift of Dr. D. MacLennan) was excised from PBS using KpnI and SacI, and for the CHOP probe, a mouse cDNA (gift of Dr. D. Ron) was excised from PBS using EcoRI.

**RESULTS**

**Expression of calcium regulatory ER stress proteins.** The upregulation of specific ER calcium-binding proteins is a necessary survival response to ER stress (16). After 7 days of unloading, there was a 30% loss in soleus muscle mass, but there was no difference in the relative expression of classic ER luminal stress-response proteins calreticulin (Fig. 1A) and Grp78 (Fig. 1B). Vinculin expression was also unchanged (Fig. 1C), the expression of which is known to be highly sensitive to changes in calreticulin expression during the regulation of cell adhesion (10). ER calcium-depletion stress by thapsigargin also leads to an upregulation of type I IP3R (20), but there was no change in type I IP3R expression in atrophied muscle (Fig. 1D). It is noted, though, that in earlier work, we not only demonstrated an eightfold upregulation of the skeletal-specific SERCA1 gene with unloading, but, in that same study, we found that expression of the ubiquitous SERCA2b ER stress-response gene doubled (24). SERCA2b has recently been identified as a ubiquitous ER stress-response gene (8).

**Fig. 2.** Expression of the antioxidant protein heme oxygenase-1 (HO-1). Relative expression was measured by immunoblot (A) in 30 μg of microsomal protein from control and 7-day hindlimb-unloaded soleus muscles using an HO-1 antibody. Positive control for HO-1 was recombinant rat (rr) HO-1. Blots were quantified by densitometry (B). Data are means ± SE of 4 muscles per group.

**Fig. 3.** No differences were found in mRNA or protein expression of the proapoptotic ER stress markers CHOP or protein kinase R (PKR) with unloading. A: Northern analysis of control and 1-day hindlimb-unloaded soleus muscles. For comparison, extensor digitorum longus (EDL) muscles express higher levels of CHOP mRNA. There was no difference in CHOP mRNA expression at 7 days of unloading (not shown). Blot was reprobed with α-actin for quantification. CHOP (B) and PKR (C) protein expression was measured by immunoblot in 30 μg of whole cell lysate from control and 7-day hindlimb-unloaded soleus muscles using CHOP- and PKR-specific antibodies. Positive control for CHOP was serum-starved 3T3 cell lysate, and PC-12 cell lysate was positive control for PKR. There was no difference in CHOP protein expression at 1 day of unloading (not shown). At least 4 muscles per group were assessed.
Expression of the ER antioxidant stress protein HO-1. To determine whether unloaded muscle is characterized by ER stress, as reflected by expression of an antioxidant ER stress protein, we measured HO-1 in microsomes from control and unloaded muscle. HO-1 expression was increased 2.2-fold in unloaded muscle (Fig. 2).

Expression of the transcription factor CHOP: a prototypical ER stress marker. The transcription factor CHOP/GADD-153 has been established as a strong marker of ER stress. However, expression of the mRNA (Fig. 3A) or the protein (Fig. 3B) was not changed due to unloading at either 1 or 7 days. An early time point was examined because CHOP activation is known to be a rapid event after ER stress (16). There was, however, a fiber-type difference in mRNA expression with higher levels in the fast-twitch EDL muscle compared with the slow-twitch soleus muscle.

Expression of the eIF-2α kinase PKR. Besides the activation of CHOP in response to ER stress, a second proapoptotic event in response to ER stress is the inhibition of protein translation by the phosphorylation of eIF-2α (16). We recently demonstrated that eIF-2α phosphorylation is not activated by muscle unloading (12). This is consistent with the lack of induction of the major eIF-2α kinase, PKR (Fig. 3C), which is commonly activated by ER stress (16).

Calsequestrin expression: a skeletal muscle-specific SR calcium-binding protein. Although the classic ER stress-response calcium-binding proteins were not different with 7 days of muscle unloading, the high-capacity, moderate-affinity, skeletal muscle-specific calcium-binding protein calsequestrin was increased by 68% and 24% at the mRNA (Fig. 4A) and protein (Fig. 4B) levels, respectively. This is consistent with, but not as marked as, the increase in other muscle-specific SR calcium-handling genes previously reported (14, 15, 24, 28). Together, the upregulation of SERCA1 (24, 28), SERCA2b (24), and the type I ryanodine receptor (15) by muscle unloading shown previously, and the increase in calsequestrin shown here, may be reflective of a muscle-specific SR stress response.

DISCUSSION

Skeletal muscle has the specialized function of contraction, the activation of which requires significant intracellular calcium flux. To accommodate this function, muscle has a highly developed ER (the SR). The proteins regulating intracellular calcium flux from the ER are responsible for maintaining calcium homeostasis in the cell.

Table 1. Comparison of ER stress protein markers to muscle-specific SR proteins during disuse atrophy

<table>
<thead>
<tr>
<th>ER Ca²⁺ homeostasis</th>
<th>Antioxidant</th>
<th>Transcription factor</th>
<th>Translational inhibition</th>
<th>SR Ca²⁺ homeostasis</th>
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<tr>
<td>Survival</td>
<td>Death</td>
<td>Survival?</td>
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<td>ER</td>
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<td>→ Calreticulin</td>
<td>↑ HO-1</td>
<td>→ CHOP</td>
<td>→ eIF-2α-P²⁺</td>
<td>↑ Calsequestrin</td>
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<td>→ Grp78</td>
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*See Ref. 12; † see Ref. 28; ‡ see Ref. 15; § see Ref. 24. ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; HO-1, heme oxygenase-1; eIF-2α, eukaryotic initiation factor; Grp78, 78-kDa glucose-regulated protein; PKR, protein kinase R; SERCA1, sarco(endo)plasmic reticulum Ca²⁺-ATPase; IP₃R, D-myo-inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor; SERCA2b, ubiquitous ER Ca²⁺-ATPase gene.
ER in nonmuscle are expressed at much lower levels, yet they play a central role in differential gene expression, cell growth, and cell death (4). For instance, in response to various types of cellular stresses, such as ER calcium store depletion, there are increases in the expression of resident ER proteins that have roles in maintaining intracellular calcium homeostasis (e.g., SERCA2b and ER calcium-binding proteins). This response is required for adaptation to the stress and is referred to as an ER stress response (8, 16).

Skeletal muscle disuse atrophy is associated with a marked upregulation of major SR calcium regulatory proteins such as SERCA1 (24, 28) and the type I ryanodine receptor (15) and a moderate increase in calsequestrin and the ubiquitous SERCA2b isoform (24). The increase in the capacity for the release, storage, and uptake of intracellular calcium in the face of muscle wasting appears similar to the prototypical ER stress response characterized by upregulation of the ER calcium pump (SERCA2b) (8) and ER calcium-binding/chaperone proteins (16). ER calcium-depletion stress is also characterized by increased expression of the \( \text{IP}_3 \text{R} \), an ER calcium-release channel (20). However, in the present work, the ubiquitous ER calcium-binding/chaperone proteins (or the \( \text{IP}_3 \text{R} \)) were not upregulated with disuse atrophy. Prototypical ER stress proteins having proapoptotic effects such as the transcription factor CHOP and the major eIF-2 kinases, PKR, were also unchanged. On the other hand, muscle unloading led to the activation of HO-1, an antioxidant ER stress protein. The data in Table 1 summarize the changes in the expression of ER stress proteins during muscle unloading measured in the present study and in earlier work. A comparison is made to muscle-specific SR protein expression, which is increased with muscle unloading. Expression of only two of the same genes is upregulated in both ER stress and muscle unloading, HO-1 and SERCA2b.

A potential role for the unloading-induced upregulation of muscle-specific calcium-handling proteins may be a muscle-specific stress response. Although there is no evidence that atrophied muscle has depleted SR calcium stores, as is often the case with ER stress, the increased expression of muscle-specific SR proteins might represent a survival response in the face of atrophy and disturbed intracellular calcium. Resting muscle fibers from unloaded solei have elevated free cytosolic calcium concentrations (13), suggesting a perturbation in intracellular calcium homeostasis. Moreover, SR calcium uptake (28, 30), caffeine-induced SR calcium release (30), and calcium leakage (30) are increased in unloaded, compared with control, muscle. Elevated intracellular calcium is a known trigger of proteolytic and apoptotic pathways, and overexpression of the antiapoptotic protein Bcl-2 blocks calcium-induced apoptosis (27). In other work, we have shown that Bcl-2 protein expression is strongly upregulated after 7 days of unloading (unpublished observations). Overexpression of Bcl-2 has been shown to increase SERCA2b expression in epithelial cells, helping to moderate intracellular calcium levels (19). These observations are consistent with the idea that an increase in the SR calcium-handling proteins may be part of a survival response in atrophying muscle, analogous to the ER stress response leading to cell adaptation and survival (see Table 1). Muscle, which is both postmitotic and multinucleated, may have mechanisms to promote survival during withdrawal of mitogenic stimuli, such as that associated with muscle disuse. In mitotic cells, withdrawal of mitogenic stimuli leads to apoptosis (26). If muscle cells were lost due to atrophy, there is no adequate mechanism to replace large numbers of muscle fibers.

The induction of HO-1 during unloading may also represent a defensive response to ensure muscle cell survival during atrophy. HO-1 has been shown to be activated by oxidative stress in muscle cells (5), and elevated levels of HO-1 have been shown to significantly inhibit apoptosis in several cell types (6, 11, 25, 29). This is thought to be the result of an increased carbon monoxide concentration, a product of the heme oxygenase reaction (6) and/or increased disposal of free iron (11) following ferritin induction. Increased expression of HO-1 also promotes an antioxidant response via the production of the antioxidant bilirubin and the removal of prooxidant heme. This response may attenuate the extent of oxidative damage that occurs in immobilized rat soleus muscle (17, 18). Collectively, increased HO-1 expression may promote survival of the atrophied muscle cell in the face of the proapoptotic effects of elevated cytosolic-free calcium and also protect against oxidative injury. It should be noted, however, that HO-1 induction may not be the result of oxidative stress per se but the result of the changes in calcium homeostasis. In neuronal cells, depletion of ER calcium (but not elevations in cytoplasmic calcium) was shown to increase HO-1 mRNA expression (22). Further research is needed to clarify the pathway by which HO-1 is induced in atrophied muscle and to verify a potential protective role.

In summary, the present data do not support the idea of a prototypical ER stress response with muscle unloading. The increased expression of calsequestrin further strengthened the notion of a significant upregulation of the major SR calcium-handling proteins. This remodeling may accommodate increased calcium signaling and/or calcium-mobilization functions, possibly related to cell survival. The marked upregulation of the antioxidant ER stress protein HO-1 may also promote cell survival during unloading, but further work is needed to verify such a role.

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