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ENDOPLASMIC RETICULUM STRESS INDUCES MYOSTATIN PRECURSOR PROTEIN AND NF- κ B IN CULTURED HUMAN MUSCLE FIBERS: RELEVANCE TO INCLUSION-BODY MYOSITIS.

Anna Nogalska, Slawomir Wojcik, W. King Engel, Janis McFerrin, and Valerie Askanas*
Department of Neurology, USC Neuromuscular Center, University of Southern California Keck School of Medicine, Good Samaritan Hospital, Los Angeles, CA 90017

Abstract

Sporadic-inclusion body myositis (s-IBM) is the most common progressive muscle disease of older persons. It leads to pronounced muscle fiber atrophy and weakness, and there is no successful treatment. We have previously shown that myostatin precursor protein (MstnPP) and myostatin (Mstn) dimer are increased in biopsied s-IBM muscle fibers, and proposed that MstnPP/Mstn increase may contribute to muscle fiber atrophy and weakness in s-IBM patients. Mstn is known to be a negative regulator of muscle-fiber mass. It is synthesized as MstnPP, which undergoes posttranslational processing in the muscle fiber to produce mature, active Mstn. To explore possible mechanisms involved in Mstn abnormalities in s-IBM, in the present study we utilized primary cultures of normal human muscle fibers and experimentally modified the intracellular micro-environment to induce endoplasmic-reticulum (ER)-stress, thereby mimicking an important aspect of the s-IBM muscle fiber milieu. ER-stress was induced by treating well-differentiated cultured muscle fibers with either tunicamycin or thapsigargin, both well-established ER-stress inducers. Our results indicate for the first time that the ER-stress significantly increased MstnPP mRNA and protein. The results also suggest that in our system ER-stress activates NF- κ B, and we suggest that MstnPP increase occurred through the ER-stress-activated NF- κ B. We therefore propose a novel mechanism leading to the Mstn increase in s-IBM. Accordingly, interfering with pathways inducing ER-stress, NF- κ B activation, or its action on the MstnPP gene promoter might prevent Mstn increase and provide a new therapeutic approach for s-IBM and, possibly, for muscle atrophy in other neuromuscular diseases.

Keywords

Myostatin precursor protein; myostatin; endoplasmic reticulum stress; inclusion-body myositis; Nuclear factor- κ B; cultured human muscle fibers; proteasome inhibition

Introduction

Sporadic-inclusion body myositis (s-IBM) is the most common muscle disease associated with aging (recently reviewed in Engel and Askanas, 2006). It is a relentlessly progressive disease, manifested by pronounced muscle weakness and wasting, leading to severe disability. There is no successful treatment (Engel and Askanas, 2006). Histological hallmarks of s-IBM muscle

*Corresponding author: Valerie Askanas, MD, PhD, USC Neuromuscular Center, Good Samaritan Hospital, 637 S. Lucas Ave, Los Angeles, CA 90017. Tel. 213-975-9950. Fax.213-975-9955. e-mail: askanas@usc.edu

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fibers include: a) vacuolar degeneration and atrophy of muscle fibers, accompanied by intra-muscle-fiber accumulations of congophilic, ubiquitinated, multi-protein aggregates; and b) lymphocytic inflammation (Askanas and Engel, 2006; Dalakas, 2006). The pathogenesis of s-IBM appears complex and multifactorial (Askanas and Engel, 2006). An intriguing aspect of the s-IBM muscle-fiber phenotype is its similarity to Alzheimer-disease brain, including accumulations of amyloid- β (A β), and of phosphorylated tau in the form of paired helical filaments (reviewed in Askanas and Engel, 2001; Askanas and Engel, 2006). Endoplasmic reticulum (ER)-stress and the unfolded protein response (UPR) were recently demonstrated in s-IBM muscle fibers (Vattemi et al., 2004). Further evidence of ER-stress involvement in the s-IBM pathogenesis is the increased mRNA and protein of Herp, a novel ER-stress-induced protein (Nogalska et al., 2006). Inhibition of 26S proteasome activity and the presence of aggresomes were also recently demonstrated within s-IBM muscle fibers (Fratta et al., 2005). Previously, NF- κ B was shown to be an aspect of the s-IBM pathology, but its activation has not been studied (Yang et al., 1998).

We recently showed that in s-IBM muscle fibers both myostatin precursor protein (MstnPP) and myostatin (Mstn) dimer were significantly increased, and we proposed that this increase might contribute to the muscle fiber atrophy of s-IBM (Wojcik et al., 2005).

Mstn, a member of the transforming growth factor- β (TGF- β) superfamily, is a negative regulator of muscle growth during development, and of muscle mass in adulthood (reviewed in Gonzales-Cadavid and Bhasin, 2004; Joulia-Ekaza and Cabello, 2006). A child, who has a homozygous MstnPP gene mutation resulting in reduced production of Mstn protein, has increased muscle bulk and strength (Schuelke et al., 2004). Conversely, mature Mstn protein was reported increased in muscle tissue of patients with HIV-associated muscle wasting (Gonzales-Cadavid et al., 1998), and increased MstnPP mRNA was reported in muscle wasting associated with osteoarthritis (Reardon et al., 2001). In mouse models, knocking out the myostatin gene, overexpressing proteins neutralizing myostatin, or natural mutations of the myostatin gene cause increased muscle mass (Gonzales-Cadavid and Bhasin, 2004). Accordingly, diminishing the action of myostatin might be considered a potential method for treating muscle atrophy in various neuromuscular disorders.

Within muscle fibers, Mstn is synthesized as Mstn precursor protein (MstnPP) (Gonzales-Cadavid and Bhasin, 2004; McFarlane et al., 2005). MstnPP, a 375 amino acid protein translated from a 3.1-kb mRNA (Gonzales-Cadavid et al., 1998), undergoes a series of intracellular posttranslational modifications in order to produce mature, active Mstn (Gonzales-Cadavid and Bhasin, 2004; McFarlane et al., 2005).

One of our approaches to explore molecular pathogenic mechanisms in s-IBM involves experimentally modifying the cellular micro-environment of cultured human muscle fibers (CHMFs) to mimic various aspects of the s-IBM pathogenesis (Askanas et al., 1996; 1997; McFerrin et al., 1998; Fratta et al., 2005; Nogalska et al., 2006). To address possible mechanisms involved in Mstn increase in s-IBM, in the present study we induced ER-stress in CHMFs and studied its influence on: a) expression of MstnPP mRNA and protein, and b) NF- κ B activation.

Our rationale was that: a) both ER-stress and NF- κ B are components of the s-IBM pathogenesis (Yang et al., 1998; Vattemi et al., 2004; Nogalska et al., 2006); b) ER-stress has been reported to activate NF- κ B in other cells (Pahl and Baeuerle, 1995; Pahl, 1999; Deng et al., 2004); and c) the MstnPP gene promoter has been reported to have an NF- κ B binding site (Ma et al., 2001). In our human muscle culture model, we here report for the first time that: a) ER-stress induces both MstnPP mRNA and its protein, and b) the induction of Mstn might occur through

the NF- κ B activated by the ER-stress. We also demonstrate that NF- κ B is activated in s-IBM muscle biopsies.

EXPERIMENTAL PROCEDURES

Cultured Human Muscle Fibers

Primary cultures of normal human muscle were established, as we described, from satellite cells of portions of diagnostic muscle biopsies from patients who, after all tests were performed, were considered free of muscle disease (Askanas and Engel, 1992). Experiments were performed on eight culture sets, each established from satellite cells derived from a different muscle biopsy. All experimental conditions were studied on sister cultures in the same culture set. 20 days after fusion of myoblasts, well-differentiated myotubes were treated for 24 hours with two well-known ER stress inducers, Tunicamycin, an N-glycosylation inhibitor (4 μ g/ml), or Thapsigargin, an inhibitor of ER calcium-ATPase (300 nM) (Back et al., 2005; Lee, 2005) (both inhibitors from Sigma Co, St. Louis, MO). After treatment, experimental cultures and their untreated-sister-controls were processed for: RNA isolation, immunoblotting, and isolation of nuclei for electrophoretic mobility shift assay (EMSA). In addition, treatment with two well-established NF- κ B inhibitors, Bay 11-7082, an inhibitor of I κ B phosphorylation and its subsequent degradation (10 μ M) (Pierce et al., 1997; Epinat and Gilmore, 1999), or pyrrolidine dithiocarbamate (PDTC), an inhibitor of I κ B polyubiquitination and its subsequent degradation (50 μ M) (Epinat and Gilmore, 1999; Hayakawa et al., 2003) (both from Sigma), were applied for 24 hours simultaneously with the ER-stress inducers, and to control untreated-cultures. To determine whether any of the treatments induced apoptosis in CHMFs, immunoblots of all groups were performed using a polyclonal antibody against activated caspase 3 (Santa Cruz, CA), diluted 1:200.

RNA isolation and RT-PCR

Total RNA was isolated as described, using RNA-bee reagent (Tel-Tech, Friendwood, TX) (Nogalska et al., 2006). 1 μ g of RNA was used in the RT-PCR reaction utilizing OneStep RT-PCR kit (Qiagen, Valencia, CA) and primers for myostatin (Fw-GTGGTACCTCATGCAAAAAGTCAACTCTGT; Rv -ATGGATCCAA TCTCATGAGCACCCACAGC) or for GAPDH (Lehmann et al., 2002). The optimized conditions for myostatin were 30 min at 50°C, 15 min at 95°C, followed by 31 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 90 s). The final incubation was 10 min at 72°C. In addition, 1 μ g of RNA was submitted for cDNA synthesis as described (Nogalska et al., 2006), and used in PCR reactions with specific primers obtained from Invitrogen that allow amplification of the ER-chaperone GRP78 (Lee, 2005), ER-protein Herp (Nogalska et al., 2006; Kokame et al., 2000), and XBP-1 (Back et al., 2005). The conditions of the reactions were experimentally checked to ensure that signals were in the linear range of the PCR. All experiments were performed in duplicate. Identity of the products was confirmed by sequencing. GAPDH was used as a loading control for the PCR reactions. Evaluation of PCR bands was performed by densitometric analysis using NIH Image J 1.310 software.

Immunoblotting

CHMFs were harvested in RIPA buffer, homogenized, and protein concentration measured using the Bradford method (Vattemi et al., 2004; Fratta et al., 2005). Denatured 40 μ g protein samples were loaded onto 10% NuPage gels, electrophoresed in MOPS-SDS buffer, transferred to nitrocellulose membranes, and immuno-probed overnight with anti-myostatin rabbit polyclonal antibody (Chemicon, Temecula, CA), diluted 1:500. This antibody has been shown to be highly specific in studies by ourselves and others (Gonzales-Cadavid et al., 1998; Reisz-Poraszcz et al., 2003; Wojcik et al., 2005). On immunoblots, this antibody recognizes both a 55kDa band of MstnPP and a 26kDa band of mature Mstn dimer in biopsied adult muscle

fibers, and a 55kDa band in cultured muscle fibers (McFarlane et al., 2005; and our results herein). After incubation with an appropriate secondary antibody, blots were developed using the enhanced chemiluminescence system. Protein loading was evaluated by actin bands visualized with a mouse monoclonal anti-actin antibody (1:2000) (Santa Cruz). Quantification of immunoreactivity was performed by densitometric analysis using the Kodak-GelLogic-440 system (Eastman Kodak Company, Rochester, NY).

Electrophoretic mobility shift assay (EMSA)

This was performed on nuclei isolated from CHMFs, and from s-IBM and normal-control muscle biopsies.

A. Nuclear extracts were prepared from CHMFs as described for other cells (Li et al., 2005), with some modifications. In brief, CHMFs grown in 35mm dishes were washed twice with PBS. After addition of 100 μ l of cold buffer (100mM HEPES pH7.9, 10mM KCl, 10mM EDTA, 1mM DTT, 0.4% NP-40) containing phosphatase (Pierce, Rockford, IL) and protease (Roche Diagnostic, Mannheim, Germany) inhibitor cocktails, cells were incubated 10 minutes on ice, and then scraped and disrupted by pipetting and homogenization. The lysates were centrifuged for 3 min at 14,000 rpm at 4°C. The pellet was resuspended in 20mM HEPES pH7.9, 200mM NaCl, 1mM EDTA, 10% glycerol, 1mM DTT and phosphatase and protease inhibitor cocktails, by vortexing for 60 seconds. The samples were kept on ice and repetitively vortexed for 60sec every 5 min on the maximum setting, and then centrifuged for 5min at 14,000 rpm at 4°C. The supernatants (nuclear fraction) were immediately transferred to new pre-chilled tubes. Double-stranded oligonucleotides, representing consensus sequences of either NF- κ B or Octomer -1 (OCT-1) (an irrelevant ubiquitous transcription factor used as a loading control [Jiang et al., 2003]) (both from Promega, Madison, WI), were labelled with [γ -³²P]ATP (3000Ci/mmol; Perkin Elmer, Boston, MA) using reagents provided in the Gel Shift Assay System (Promega). 5 μ g of nuclear protein isolated from CHMFs was incubated for 30 min at room temperature in 1x EMSA binding buffer (Pierce) with addition of glycerol (2.5%), Poly (dI-dC) (50 ng/ μ l), NP-40 (0.05%), MgCl₂ (5mM), in a final assay volume of 20 μ l. DNA/protein complexes were separated by gel electrophoresis in 6% DNA retardation gel (Invitrogen).

B. EMSA for translocated nuclear NF- κ B was performed on nuclear extracts prepared from three control and three s-IBM muscle biopsies, as described above for CHMFs. The results were densitometrically scanned and calculated per OCT-1 as a loading control.

Statistical analysis

The statistical significance was determined by ANOVA followed by the Tukey-Kramer post-hoc test, using GraphPad InStat software. Statistical significance of differences between s-IBM and control muscle biopsies (comparing two groups) was determined by Student T-test. The level of significance was set at $p < 0.05$. Data are presented as means $\pm$ SEM for all groups.

RESULTS

Treatment with tunicamycin or thapsigargin induces ER-stress in CHMFs

We used 24 hours of treatment with 4 μ g/ml tunicamycin (Tm) or 300 μ M thapsigargin (Tg) in all experiments, since in our previous study (Nogalska et al., 2006) those doses and the timing were the most effective, and they did not induce any adverse morphological abnormality or affect muscle-fiber viability. Because human muscle material from which those primary cultures are derived is very sparse, we were not able to perform ranges of doses. In the present study, treatment of CHMFs with Tg or Tm induced characteristic molecular markers of ER-stress such as: a) XBP-1 mRNA splicing; b) increase of GRP78 mRNA; and c) increase of Herp mRNA (Fig. 1A and 1B).

ER-stress induces MstnPP mRNA and protein in CHMFs

24 hours of treatment with either ER-stress inducer greatly increased MstnPP mRNA (Fig. 2A, B). (Because CHMFs treated with Tg for either 6 or 24 hours had similarly increased MstnPP mRNA level, 24 hour-treatment was used in all experiments.) In addition to the increased MstnPP mRNA, treatment with either Tg or Tm significantly increased MstnPP protein (Fig. 2C, D). (Mature 13kDa myostatin and 26kDa myostatin dimer were not apparent on our immunoblots, which is consistent with a recent study demonstrating that in well-differentiated myotubes of a C2C12 mouse-muscle cell-line and bovine myotubes, mature myostatin was not detected on immunoblots [McFarlane et al., 2005]).

ER-stress activates NF- κ B in CHMFs

To evaluate whether ER-stress activates NF- κ B in our CHMFs, we studied the induction of NF- κ B DNA-binding activity by EMSA performed on nuclear extracts prepared from cultures treated with Tg or Tm, as compared to the unstressed-sister-control cultures. NF- κ B DNA-binding in CHMFs was dramatically increased in cultures treated with either Tg or Tm. As a loading control, we measured DNA-binding of a ubiquitous transcription factor OCT-1 (Fig. 3A). The specificity of the NF- κ B DNA-binding was confirmed by adding to the binding mixture either: a) a 100x molar excess of a specific cold unlabeled DNA probe containing the NF- κ B consensus sequence (Fig. 3B, sp), or b) an excess of a non-specific cold CREB DNA probe containing the ATF consensus binding sequence (Jiang et al., 2003) (Fig. 3B, nsp). Only the specific probe containing the NF- κ B consensus binding sequence eliminated the DNA-NF- κ B binding (Fig. 3B, sp). Simultaneous treatment of CHMFs with either of the ER-stress inducers and a known NF- κ B inhibitor, Bay 11-7082 or PDTC, resulted in no shifted band being present (Fig. 3C), which suggested an inhibition of NF- κ B activation. Together, the above indicate that, similarly to other cells, in cultured human muscle fibers NF- κ B is induced by ER-stress.

NF- κ B participates in ER-stress-induced increase of MstnPP mRNA

To evaluate possible involvement of NF- κ B in transcriptional regulation of Mstn mRNA expression during ER-stress, we cultured human muscle fibers in the presence of each of the ER-stress inducers and each NF- κ B inhibitor, and evaluated their influence on MstnPP mRNA. Treatment with an NF- κ B inhibitor, either Bay 11-7082 or PDTC, did not influence morphology or viability, or lead to apoptosis of either control or ER-stress-induced CHMFs. Lack of apoptosis was evidenced by lack of caspase-3 cleavage on the immunoblots (Fig. 3D). However, in contrast to the control-unstressed-sister cultures in which NF- κ B inhibitors had no effect on MstnPP mRNA (Fig. 4A, B), simultaneous treatment of CHMFs with either ER-stress inducer, Tg or Tm, and either NF- κ B inhibitor profoundly inhibited the MstnPP mRNA increase obtained by treatment with either ER-stressor alone (Fig. 4C, D). Those results strongly suggest NF- κ B involvement in the regulation of Mstn mRNA during ER-stress. To evaluate the specificity of the observed response, we studied the influence of the NF- κ B inhibitors on GRP78 and Herp mRNAs in the same cultures in which MstnPP mRNA was studied. In contrast to MstnPP mRNA, simultaneous treatment of CHMFs with the ER-stress-inducer thapsigargin and NF- κ B inhibitor Bay 11-7082, increased GRP78 but not Herp mRNAs, while treatment with tunicamycin and Bay 11-7082 did not cause any significant changes in expression of GRP78 and HERP mRNAs (Fig. 4E–H). This suggests a distinct regulation of the MstnPP gene from those of GRP78 and Herp. However, the other NF- κ B inhibitor, PDTC, decreased GRP78 and Herp mRNAs in tunicamycin treated cultures (Fig. 4E–H), but to a lesser degree than it decreased Mstn mRNA studied in the same cultures (Fig. 4C–D). Since, in addition to inhibiting NF- κ B activation, PDTC has also been shown to inhibit activity of the 26S proteasome (Kim et al., 2004; Si et al., 2005), in two independent experiments we simultaneously treated CHMFs for 24 hours with either of the ER-stress

inducers together with 1 μ M epoxomicin (Biomol Research Laboratories, Plymouth Meeting, PA), an irreversible proteasome inhibitor (Meng et al., 1999). In our previous studies, 24 hours of treatment with this dose of epoxomicin did not affect muscle morphology or viability, but it effectively inhibited proteasome activity (Fratta et al., 2005; Nogalska et al., 2006). In the present study, similarly to the PDTC effect, epoxomicin treatment prominently decreased MstnPP mRNA, and to a lesser degree also decreased mRNAs of GRP78 and Herp (Fig. 5A). Moreover, as previously reported for other proteasome inhibitors tested in other cells (Epinat and Gilmore, 1999), in our studies treatment with epoxomicin also inhibited NF- κ B DNA-binding activity in CHMFs (Fig. 5B).

NF- κ B activation is increased in s-IBM muscle fibers

EMSA performed on nuclei isolated from s-IBM and control muscle biopsies showed 4-fold ($p < 0.05$) increase of NF- κ B binding in s-IBM muscle biopsies as calculated by a densitometric analysis of NF- κ B per OCT-1 (Figs. 6A and 6B).

DISCUSSION

Our study provides novel results suggesting that MstnPP gene expression is regulated at least partly by ER-stress. Additionally, our results suggest that, in human cultured muscle fibers, increased expression of MstnPP occurs through ER-stress-induced activation of transcription factor NF- κ B. The mechanism of ER-stress-increased MstnPP mRNA appears to be different from the ER-stress-induced increase of mRNAs of the two well-known ER-stress-induced proteins: GRP78 and Herp, since a specific NF- κ B inhibitor, Bay 11-7082, greatly reduced MstnPP mRNA abundance but did not have a similar influence on GRP78 and Herp mRNAs. Another NF- κ B inhibitor, PDTC, which is known to also inhibit proteasome function (Kim et al., 2004; Si et al., 2005), decreased not only MstnPP mRNA, but also mRNAs of GRP78 and Herp. It has been previously reported that proteasome inhibition disturbs ER function, and it reduces activation of the ER-stress-induced chaperone GRP78 (Lee et al., 2003) and Herp (Nogalska et al., 2006). It has also been reported that experimental proteasome inhibition reduces activation of NF- κ B (Epinat and Gilmore, 1999; Hayakawa et al., 2003). Our study demonstrates for the first time that proteasome inhibition greatly decreases ER stress-induced Mstn mRNA, and we propose that this response is obtained through preventing NF- κ B activation by ER-stress. Activation of NF- κ B during ER-stress in other cells has been shown to occur through the ER-overload response (EOR) (Pahl and Baeuerle, 1997), whose signaling pathways were proposed to be distinct from the unfolded protein response (UPR) (Pahl and Baeuerle, 1997; Jiang et al., 2003; Hung et al., 2004). Although several pharmacological and biological agents induce both NF- κ B activation and UPR, some of the inducers distinguish between the two pathways (Pahl and Baeuerle, 1997). NF- κ B is a ubiquitous, inducible transcription factor, involved in many important biological processes, including inflammation and apoptosis. In non-stimulated cells, NF- κ B resides in the cytoplasm where it is bound to its inhibitory protein I κ B (Grossmann et al., 1999). In response to various stimuli, NF- κ B dissociates from I κ B and then translocates into the nucleus, where it binds to the promoters of various genes to influence their expression (Grossmann et al., 1999; Schmitz et al., 2004). NF- κ B proteins can exist as homo- or hetero-dimers, composed of any of five different subunits, p50 (NF- κ B1), p52 (NF- κ B2), p65 (RelA), RelB and c-Rel (Grossmann et al., 1999; Schmitz et al., 2004). We propose two possible mechanisms of MstnPP upregulation by ER-stress-activated action of NF- κ B: a) a direct mechanism, in which NF- κ B binds to its reported binding sequence in the MstnPP gene promoter, or b) an indirect mechanism, in which NF- κ B binds to an NF- κ B consensus site in the promoter of another gene(s) that encodes a protein(s) involved in MstnPP regulation. The 5'-regulatory region of human MstnPP gene is known to contain various transcriptional response elements. Among them an NF- κ B consensus site was identified, but no testing of its functionality was performed (Ma et al., 2001). There is no

published evidence suggesting presence of any known ER-stress response elements in the promoter region of MstnPP gene. Further experimental studies are needed to elucidate the precise mechanism of the ER-stress-induced NF- κ B influencing MstnPP regulation. Whether calcium release from the sarcoplasmic reticulum, p38 MAPK activation, or other pathways previously shown to activate NF- κ B during ER stress (Pahl and Baeuerle, 1996; Jiang et al., 2003; Deng et al., 2004; Hung et al., 2004; Hu et al., 2006) participate in the NF- κ B induction and MstnPP mRNA increase in cultured human muscle fibers remain to be studied.

Previously, it was reported that TNF α -induced-NF- κ B does not influence MstnPP mRNA in a C2C12 mouse muscle cell line (Bakkar et al., 2005). Similarly, in cultured human muscle fibers, we did not observe any changes in either MstnPP, or GRP78 and Herp mRNA levels when NF- κ B was induced by treatment with TNF α (data not shown). While those results seem contradictory to our demonstration here that NF- κ B activation increases Mstn mRNA, the difference might be explained by: a) the fact that under different stimulatory situations different homo- and hetero-dimer combinations of NF- κ B take place, which subsequently may result in different NF- κ B binding properties to its DNA targets (Kunsch et al., 1992; Pahl 1999; Jiang et al., 2003); or b) TNF α might have a separate, direct or indirect, inhibitory effect on activation of the MstnPP gene. Furthermore, different NF- κ B co-activators or posttranslational modifications of its subunits might also influence its different binding properties (Rahman et al., 2004; Schmitz et al., 2004; Viatour et al., 2005).

Previously, NF- κ B accumulation was shown in s-IBM muscle fibers (Yang et al., 1998), but its nuclear translocation was not studied. Our present study demonstrates that there is increased NF- κ B bound to nuclear DNA, suggesting increased activation of NF- κ B in s-IBM muscle fibers. Several factors proposed to participate in the s-IBM pathogenesis, including ER-stress, inflammation, and accumulation of amyloid- β (Askanas and Engel, 2006; Dalakas, 2006), might contribute to the NF- κ B activation in s-IBM muscle fibers (Pahl and Baeuerle 1995; Pahl 1999; Deng et al., 2004; Kaltschmidt et al., 2005). Here, based on our experimental studies, we suggest that ER-stress causes NF- κ B activation and increase of MstnPP in cultured human muscle fibers. Since ER-stress has been demonstrated in s-IBM muscle biopsies, the same mechanism might also be occurring in s-IBM patients' muscle fibers to explain their demonstrated increase of MstnPP and Mstn (Wojcik et al., 2005). The increased Mstn is likely contributing to the prominent muscle-fiber atrophy in s-IBM. Although the exact mechanisms involved in the ER-stress induction of MstnPP mRNA in human muscle fibers still need to be elucidated, our data suggest that interfering with the induction of, or effects of, ER-stress might prevent Mstn increase and provide new therapeutic approaches for s-IBM. Whether this mechanism contributes to muscle atrophy in other neuromuscular disorders needs to be evaluated.

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Fig 1. Treatment with Tg and Tm induce ER-stress in cultured human muscle fibers (CHMFs)
Representative agarose gel electrophoresis of XBP-1 (A), GRP78 and Herp (B) PCR products in control “C” and in Tg and Tm treated CHMFs. They illustrate evidence of the ER-stress by: XBP-1-splicing, and increased GRP78 and Herp mRNAs. GAPDH mRNA is used as a loading control. MW – DNA molecular weight marker.

Fig 2. ER-stress induces MstnPP mRNA and protein in CHMFs

(A) Representative agarose gel electrophoresis of PCR products corresponding to myostatin precursor protein (MstnPP) mRNA obtained from control “C” cultured human muscle fibers (CHMFs), and CHMFs treated for 24 h with ER-stress inducers thapsigargin (Tg) or tunicamycin (Tm). GAPDH mRNA was used as a loading control. (B) Densitometric analysis of the PCR bands, performed using NIH Image J 1.310, and expressed in arbitrary units based on eight independent experiments. The GAPDH mRNA was used to normalize corresponding MstnPP mRNA bands. Control is designated as “1”. (C) Immunoblots of homogenates of control CHMFs and CHMFs, treated with Tg or Tm, showing 55kDa MstnPP. The actin bands indicate protein loading in each sample. (D) Densitometric analysis of the bands performed with the Kodak-GelLogic-440 system, based on 8 independent experiments. Data are expressed in arbitrary units. $\pm$ S.E.M. “***” indicates significance of $p < 0.001$; “*” indicates significance of $p < 0.05$.

Fig 3. ER-stress activates NF-κB in cultured human muscle fibers

(A) Autoradiogram of an NF-κB EMSA performed on nuclear extracts from control “C” and Tg- and Tm-treated CHMFs. Shown below, as a loading control, is EMSA for the constitutive DNA-binding protein OCT-1 performed on the same nuclear extracts as NF-κB. (B) To determine the specificity for the NF-κB binding site, unlabeled DNA, containing either the NF-κB binding-site (sp = specific competitor) or the unrelated CREB DNA binding- site (nsp = nonspecific competitor), was added at 100x molar excess to EMSA binding mixtures containing nuclear extracts from Tm-treated cultures. Only the specific competitor eliminated NF-κB shift. (C) Autoradiograms of an NF-κB EMSA performed with nuclear extracts from CHMFs treated with Tm or Tg alone, and with addition of NF-κB inhibitors Bay 11-7082 or PDTC. Both inhibitors essentially eliminated the NF-κB shift. (D) Using antibody against caspase-3, immunoblot of homogenates of control CHMFs and CHMFs undergoing various treatments shows lack of apoptosis as evidenced by lack of 20 and 17 kDa bands that would indicate cleaved forms of caspase-3. Those bands are clearly visible in Jurkat cells undergoing apoptosis due to treatment with cytochrome c (J act), but are not present in untreated-control Jurkat cells (J). Control and cytochrome c-treated Jurkat cells (human leukemia T-cells) were obtained from Cell Signaling (Danvers, MA).

Fig 4. NF-κB participates in ER-stress-induced increase of MstnPP mRNA

(A) Representative agarose gel electrophoresis of the RT-PCR products corresponding to MstnPP in control "C" CHMFs, and in control cultures treated with ER-stress inhibitors Bay 11-7082 or PDTC. GAPDH was used as a loading control. NF-κB inhibitors do not influence MstnPP mRNA in control CHMFs (A and B). (C) Representative agarose gel electrophoreses of the RT-PCR products corresponding to the MstnPP mRNA from cultures simultaneously treated with either Tg and an NF-κB inhibitor Bay 11-7082 or PDTC, or with Tm and either of NF-κB inhibitor, as compared to cultures treated with either Tg or Tm alone, showed prominent decrease of MstnPP mRNA. (D) Densitometric analysis of the PCR bands as in (C), based on 8 experiments and expressed in arbitrary units, indicates that, compared to CHMFs treated with either Tg or Tm alone, simultaneous treatment of cultures with Tg plus either NF-κB inhibitor, or with Tm plus either NF-κB inhibitor, greatly decreased MstnPP mRNA (** = $p < 0.001$). (E and G) Representative agarose gel electrophoreses of the RT-PCR products corresponding to GRP78 (E) or Herp (G) mRNAs from cultures simultaneously treated with Tg plus either NF-κB inhibitor, Bay 11-7082 or PDTC, or with Tm plus either NF-κB inhibitor, as compared to cultures treated with either Tg or Tm alone. (F and H) Densitometric analyses of the PCR bands as in E and G, based on 8 experiments and expressed in arbitrary units, indicate that, cultures treated simultaneously with Tm and PDTC, had decreased mRNAs of both GRP78 and Herp, even though to a lesser degree that the decrease of MstnPP mRNA (D). (** = $p < 0.001$; * = $p < 0.05$).

Fig 5. Epoxomicin decreases mRNAs of MstnPP, GRP 78 and Herp in ER-stress-induced CHMFs, and it eliminates the NF-κB shift in them

(A) Representative agarose gel electrophoreses of the PCR products corresponding to MstnPP, GRP78 and Herp mRNAs from cultures simultaneously treated with either Tg or Tm combined with epoxomicin, or with Tg and Tm alone. There is a profound decrease of MstnPP mRNA, and a slightly smaller decrease of GRP78 and Herp mRNAs. (B) Autoradiogram of an NF-κB EMSA performed with nuclear extracts from control, Tg, and Tm treated CHMFs, as well from CHMFs simultaneously treated with the ER-stress inducer, Tg or Tm, plus epoxomicin. Addition of epoxomicin greatly reduces NF-κB activation.

Fig 6. NF-κB activation is increased in s-IBM muscle fibers

(A) Autoradiogram of an NF-κB EMSA shows increased activation of NF-κB in s-IBM. OCT-1 indicates loading control. (B) Densitometric analysis based on 3 control and 3 s-IBM muscle biopsies, normalized per OCT-1, shows greatly increased NF-κB activation in s-IBM muscle fibers (*= $p < 0.05$).