Overexpression of inducible 70-kDa heat shock protein (HSP70) in mouse attenuates skeletal muscle damage induced by cryolesion

Elen H. Miyabara¹², Jody L. Martin², Tina M. Griffin², Anselmo S. Moriscot¹*, Ruben Mestril².

¹Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, SP, Brazil and ²Cardiovascular Institute and Department of Physiology, Loyola University of Chicago, Maywood, IL, USA.

Running head: HSP70 in skeletal muscle damage

*Corresponding author:

Anselmo Sigari Moriscot

Av Lineu Prestes 1524, Departamento de Biologia Celular e do Desenvolvimento ICB I, 05508-900, Sao Paulo, SP, Brasil.

Tel\Fax: +55-11-30917311.

moriscot@usp.br
ABSTRACT

Heat shock protein expression is elevated upon exposure to a variety of stresses and limits the extent of the stress-induced damage. In order to investigate the putative role of inducible 70kDa heat shock protein (HSP70) in skeletal muscle damage and regeneration, soleus and tibialis anterior (TA) muscles from HSP70 overexpressing transgenic mice were cryolesioned and analyzed after 1, 10 and 21 days. Histological analysis showed that the muscles from both HSP70 mice and wild type mice treated with radicicol (HSP inducer) had decreased necrosis after cryolesion when compared to controls. The decrease in muscle fiber cross-section area (CSA) in both soleus and TA muscles in 10 days post lesion was attenuated in HSP70 mice when compared to wild type mice. Glutathione peroxidase (GPx) activity was increased at 1 day after cryolesion in both HSP70 and control mice and remained elevated up to 21 days. Immunodetection of neuronal cell adhesion molecule (NCAM, a satellite cell marker) and developmental/neonatal MHC (d/n) were significantly lower in cryolesioned HSP70 mice as compared to cryolesioned controls. These results suggest that HSP70 protects skeletal muscle against injury and radicicol might be useful as a skeletal muscle protective agent.

Key-words: skeletal muscle damage; regeneration; radicicol; transgenic mouse; myoprotection.
INTRODUCTION

Skeletal muscle regeneration is a highly synchronized process involving the activation of various cellular responses. After the stimulus of injury, the skeletal muscle repair is initially characterized by the necrosis and activation of an inflammatory response (Tidball 2005). This phase is rapidly followed by the activation of satellite cells (SCs), myogenic/precursor cell proliferation, differentiation and fusion to form new fibers or to restore the damaged region of the muscle fiber (Hawke and Garry 2001; Charge and Rudnicki 2004).

Although the process of the skeletal muscle damage and subsequent regeneration has been explored carefully for many years at the structural level, the identification and characterization of molecular players involved are still ongoing. Accordingly, a family of proteins known as heat shock proteins (HSPs) might be important players in skeletal muscle response to injury and subsequent regeneration, since those proteins exert a key role in cytoprotection (Mestril and Dillmann 1995; Benjamin and McMillan 1998).

The inducible 70-kDa heat shock protein (HSP70) is the most strictly stress-inducible member of the HSP family and therefore regarded as a “marker” for cell stress (Baba et al. 1998). Studies have demonstrated that the HSP70 is involved in diversified cellular functions, which include being part of the ubiquitin/proteasome system, chaperoning proteins into degradation pathways, binding of new synthesized amino acid chains on ribosomes, and maintaining translocation-competent folding of endoplasmic reticulum and mitochondrial precursor proteins in the cytosol (Pilon and Schekman 1999). Functional studies have shown that HSP70 plays a pivotal role in cardiac protection against ischemia in vivo and in vitro (Benjamin and McMillan 1998). In
addition, HSP70 is induced by certain stressors in skeletal muscle, including; physical and chemical factors; and metabolic challenges (Lindquist 1986), such as exercise-induced physiological and biochemical changes (Locke and Noble 1995).

A series of compounds able to either disrupt the chaperone complex or inhibit HSP90 are available to address the biological role of HSPs. Those compounds include the benzoquinone ansamycin antibiotics geldanamycin and herbimycin A (Morris et al. 1996; Conde et al. 1997) and, recently a more powerful compound, the macrocyclic antifungal antibiotic radicicol (Griffin et al. 2004). Ultimately, the compounds activate HSP expression by binding to the HSP 90 kDa (HSP90), which has been implicated in the regulation of the heat shock factor 1 (HSF1). HSF1 is responsible for the transcriptional activation of the heat shock genes. It has been shown that radicicol strongly induces HSP expression and subsequently ischemic protection in neonatal rat cardiomyocytes (Griffin et al. 2004).

Since the HSP70 has been recognized as tissue protector and overexpression of HSP70 in transgenic mice protects cardiomyocytes against ischemia-reperfusion injury (Marber et al. 1995) and skeletal muscles against a lengthening contraction damage model and age-related muscle disfunction (McArdle et al. 2004); we hypothesized that the HSP70 is involved in skeletal muscle protection against damage and regeneration. Therefore, we aimed to investigate the role of HSP70 in histological, immunohistochemical and molecular aspects of skeletal muscle damage and regeneration of cryolesioned HSP70 overexpressing transgenic mice. Furthermore, we evaluated the effect of the HSP inducer, radicicol, immediately following cryolesion in skeletal muscle of wild type mice.
Our results suggest that the HSP70 protects skeletal muscle against injury and that radicicol might be useful as a therapeutic myoprotective agent, since both the HSP70 overexpressing transgenic mouse and the wild type mice treated with radicicol have minimized myonecrosis induced by cryolesion.
MATERIALS AND METHODS

This study was conducted with animal care guidelines issued by the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of Loyola University Medical Center.

Animals

The transgenic mice overexpressing HSP70 were previously generated and described in detail elsewhere (Marber et al. 1995; Mestril 2005). In brief, the rat HSP70 was cloned into pCAGGS, a vector known to produce high transgenic expression utilizing a human cytomegalovirus enhancer upstream of the chicken β-actin promoter-intron. Standard methods were used to generate founders, which were screened by southern blot analysis, and bred to homozygosity. Male transgenic mice overexpressing HSP70 (n=27) and wild type mice CB6F1 (n=35) (3 months-old) weighing 22±2.5 g and 28.5±2.3 g respectively, were housed in standard plastic cages in an animal room with controlled environmental conditions and maintained on standard food and water ad libitum.

Experimental design

In all animals used in the present study, except those from radicicol control group, one soleus and one tibialis anterior (TA) muscle were cryolesioned (left leg) and the contralateral soleus and TA (right hindlimb) muscles were used as control. The animals were divided into 8 groups, i.e. 3 groups of both HSP70 mice and CB6F1 mice were cryolesioned and analyzed 1, 10 and 21 days after lesion (n=9 each), one group (n=4) was
treated with radicicol immediately after cryolesion (~2 min) and evaluated following 1 day and other group (n=4) was treated with radicicol alone for 1 day. The animals were anesthetized and the soleus and TA muscles to be lesioned were surgically exposed.

The cryolesion consisted of one freeze-thaw cycle of the muscles in situ. Freezing was performed by precisely applying the flat end piece of metal, pre-cooled in liquid nitrogen, to the surface of the muscles and maintaining it in this position for 10 seconds (1 x 9 mm and 2 x 9 mm, in soleus and TA muscles respectively). After the muscles had thawed, the wounds were closed with polyamide threads (6-0), and thereafter, the animals were kept for several hours on a warm plate (37°C) to prevent hypothermia.

**Drug treatment**

In four wild type mice, radicicol (Sigma, diluted 1:1 with ethanol) was intramuscularly injected in soleus and TA muscles from left hindlimb (0.05 mg/20 g of body weight each) immediately after the cryolesion (~ 2 min). The right hindlimb was injected with the vehicle only. Other group received treatment with radicicol alone.

**Histology**

Mice were euthanized and left and right soleus and TA muscles were removed, weighed, and half of each muscle was immediately frozen in hypercooled isopentane and stored in liquid nitrogen. Frozen muscles were cut, generating 10 µm thick entire muscle cross-sections using a cryostat (IEC Minotome). The sections were collected on gelatin-coated glass slides, dried for 1 h at room temperature and stored at –20°C. The other half of each muscle was used for immunoblotting or enzyme assay preparation.
Unfixed entire histological cross-sections were stained with aqueous toluidine-blue-borax solution (both 1% w/v) to reveal the general morphology (Morini et al. 1998; Salvini et al. 1999). The cross-sections of the cryolesioned muscles were analyzed and digitalized (Figs 2-4) only in the site of injury, therefore avoiding the muscle fibers which were not affected by cryolesion.

The regions of muscle injuries, in which active macrophages are present, were localized by the histochemical detection of lysosomal acid phosphatase activity (ac-phosphatase, Gomori lead method) according to Bancroft (Bancroft 1996). It is well known that normal muscle fibers do not have positive ac-phosphatase reaction, which indicates high concentration of lysosomes, and is considered proof of tissue necrosis and phagocytosis (Carpenter and Karpati 1984).

Antibodies for immunostaining/western blot

The primary antibodies used for immunostaining were: (1) monoclonal mouse anti-myosin heavy chain (MHC) II, clone MY-32 (1:1,000; cat# M4276, Sigma); (2) monoclonal mouse anti-skeletal myosin MHC I, clone NOQ7.5.4D (1:4,000; cat# M8421, Sigma); (3) rabbit anti-neural cell adhesion molecule (NCAM) affinity purified polyclonal antibody (2.5 µg/ml; cat# AB5032, Chemicon); (4) monoclonal mouse anti-developmental/neonatal myosin heavy chain (MHCd/n; 1:20; RNMy2/9D2, cat# VPM664, Novocastra).

The corresponding secondary antibodies used for immunostaining were: (1) goat anti-mouse IgG- fluorescein isothiocyanate (FITC) (1:50; cat# SC-2010, Santa Cruz Biotechnology); (2) goat anti-mouse IgG - FITC (1:50; cat# SC-2010, Santa Cruz
Biotechnology); (3) rhodamine red goat anti-rabbit IgG (1:50; cat# Rb394, Molecular Probes); (4) mouse IgG conjugated to horseradish peroxidase (1:20; cat# PK-6102, Vectastain Elite ABC Kit, Vector).

The primary antibodies used for western blot were: (1) an HSP70 polyclonal (1:2000) that was raised, in rabbit, against a synthetic peptide as previously described (Mehta et al. 1988); (2) mouse monoclonal anti-actin, clone C-4; (1:5,000, cat# 69100, MP Biomedicals). The corresponding secondary antibodies used for western blot were: (1) goat anti-rabbit IgG, peroxidase conjugated (1:5,000; cat# PI-1000, Vector Laboratories); (2) goat anti-mouse IgG, peroxidase conjugated (1:5,000, cat# 31430, Pierce Biotechnology).

Immunostaining

The cross-sections of muscles for immunostaining against all antibodies, except for MHCd/n, were fixed with 4% paraformaldehyde in 0.2M phosphate buffer (PB) for 10 min at room temperature, blocked with 0.1 glycine in phosphate-buffered saline (PBS) for 5 min and permeabilized in 0.2% triton X-100/PBS for 10 min. The slides were incubated with a solution containing the primary antibody, 3% normal goat serum and 0.3% triton X-100/0.1M PB overnight in a moisture chamber (4°C). After washing with 0.1M PB (3 times for 10 min each), a solution containing the secondary antibody and 0.3% triton X-100/0.1 PB were added for 2 h in a dark room. The slides were washed in 0.1M PB (3 times for 10 min each) and incubated with 3, 3’-diaminobenzidine (DAB) substrate kit for peroxidase (cat# SK-4100, Vector Labs). After washing 5 min in PBS the
slides were mounted with Vectashield mounting medium for fluorescence with 4’, 6-
diamidino-2-phenylindole (DAPI) (cat# H-1200, Vector Labs) and coverslips applied.

Unfixed muscle cross-sections were immunostained against MHCd/n antibody by
using the Vectastain Elite ABC Kit (Vector) following the manufacturer’s
recommendations. The sections were then counterstained with hematoxylin.

Observations and photography of the stained sections were made with a
fluorescent microscope (804388, Nikon, Japan) equipped with FITC, rhodamine and fura
filters or with the Radiance 2000 confocal system equipped with an inverse microscope
(Nikon Eclipse TE 300) and a scanning system (Bio-Rad). The FITC fluorochrome was
excited by an argon laser (488 nm) and the rhodamine fluorochrome were excited by
HeNe laser (543 nm). A 40x oil immersion objective was used.

Western blot

Cellular protein extracts were prepared from the muscle tissue of both control and
transgenic mice. The level of HSP70 was quantified by western blot analysis as
previously described (Griffin et al. 2004), using a specific antibody to HSP70. Protein
samples were fractionated for western blot analysis on an 8% SDS-polyacrylamide gel and
electro transferred on to nitrocellulose using a submersion electro transfer apparatus
(BioRad). The nitrocellulose blots were reacted with polyclonal antibodies that bind
specifically to HSP70 and actin. Blots were then reacted with an anti-rabbit IgG biotin-
streptavidin, horseradish peroxidase conjugated antibody and developed with ECL
developing kit (cat# 34080 Pierce).
**Determination of antioxidant enzyme activity**

To measure levels of glutathione peroxidase (GPx), frozen skeletal muscle tissues from both cryolesioned and control animals were thawed on ice. Tissues were soaked in 1 x PBS with 0.16mg/mL heparin for one minute. The tissue was removed, pulverized in liquid nitrogen and transferred to microfuge tube.

Samples for GPx activity were dounce homogenized in 10 ul/mg homogenization buffer (50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA) and 1mM dithiothreitol (DTT)- pH 7.5). They were then spun at 10,000g for 15 min at 4°C. Supernatant were transferred to new tubes and stored at -80°C until use. Assay was performed according to kit instructions (Glutathione Peroxidase Assay kit, Caymen Chemical, Ann Arbor, MI cat# 703102). Values were calculated according to the extinction coefficient of NADPH in solution, and adjusted for path length, as detailed by the manufacturer.

**Quantitative and morphometric analysis**

The quantitative and morphometric analysis were evaluated on a digitizing unit connected to a computer (Image Pro-plus, Media Cybernetic).

For the incidence of muscle fiber type I and II and their respective cross-sectional areas (CSAs), a total of approximately 500 (soleus) and 1,000 fibers (TA) per muscle per each group were counted, classified and measured after immunostaining with antibody against MHC I and MHC II in the soleus and TA muscles, respectively. Three to four cross-sections of the soleus and TA muscles from different animals were analyzed in all groups.
The number of soleus necrosed myofibers was expressed by mean percentage of 500 counted fibers per muscle (total of 1,500 fibers per group) and, for the TA muscle, as the mean percentage of 1,000 counted fibers per muscle (total of 3,000 fibers per group). Three cross-sections of the soleus and TA muscles from different animals were analyzed in all groups.

As previously described (Dedkov et al. 2003), the frequency of NCAM per muscle were expressed as a percentage of the ratio of the number of NCAM positive-SCs to the number of fibers (associated and not associated with SCs). The MHCd/n positive area was quantified and expressed as the percentage of whole muscle cross-sectional area (Duguez et al. 2003). Three whole muscle cross-sections from different animals of each group were used for both NCAM and MHCd/n analyzes.

The soleus and TA muscle fiber CSAs in different times of regeneration were obtained from a total of approximately 1,000 and 2,000 fibers, respectively. In the cryolesioned groups the CSA measurements were obtained only in the regions that were previously injured. Approximately 3-4 cross-sections of the soleus and TA muscles from different animals were analyzed in all groups.

The data from muscle weight and fiber CSAs in different times of regeneration were also presented as fold induction in order to better show the alterations over time, avoiding the influence of the initial differences in muscle weight and fiber CSAs between wild type and HSP70 mice (table 2).
Statistical analysis

Multiple comparisons of mean values were performed with analysis of variance (ANOVA) and a post-hoc Tukey’s test to compare mean values when appropriate. For comparisons between only two groups, the unpaired $t$-test was used. For all comparisons, a $p<0.05$ was considered significant.
RESULTS

Our initial goal was to confirm that transgenic mice were properly over-expressing HSP70. We also sought to determine whether cryolesion and radicicol elevated HSP70 expression in skeletal muscle from wild type mice. Our results show that in the skeletal muscle of control mice (CB6F1) cryolesioned at 1 day post-lesion there is a slight increase in the expression of HSP70 due to the injury (fig. 1a). HSP70 is highly expressed in the transgenic mice and robustly increased in the skeletal muscle of both cryolesioned at 10 days post-lesion (fig. 1b) and radicicol treated wild type mice after one day (fig. 1c).

The overexpression of HSP70 in transgenic mice reduced body weight by ~23%, soleus and TA muscle weights by 26.5% and 19.5% respectively and fiber type CSA from soleus and TA muscles by ~15% (type I; soleus), 32.5% (type II; soleus) and 14.5% (type II; TA) respectively, compared with the body and muscle weights and CSA of wild type mice (table 1). Immunostaining results revealed that both soleus and TA muscles from HSP70 mice did not have changes in MHC I/II abundance of muscle fiber type I and II when compared to the muscles from wild type mice (table 1).

Muscle weight was measured at 1, 10 and 21 days post lesion and the results show that soleus muscle weights from both wild type and HSP70 mice increased at 1 day post lesion (~80% and 60% respectively; table 2) and at 10 and 21 days after cryolesion they returned to the control values. TA muscle weights from wild type mice also significantly increased 1 day post lesion (~30%; table 2) while there was no change in TA weight in HSP70 mice 1 day post lesion. At 10 days post lesion TA muscle weights from only HSP70 mice decreased (~30%; table 2). At 21 days post lesion, TA muscle weights from
both wild type and HSP70 mice did not have significant differences when compared to their controls.

The fiber CSA was also measured at 10 and 21 days post lesion and the results indicate that, as expected, soleus muscles from wild type mice had a greater decrease in fiber CSA than those from HSP70 mice at both 10 days (55% and ~20% respectively; table 2) and 21 days after cryolesion (20% and 5% respectively, table 2). The fiber CSA of TA muscles from wild type mice decreased (~40%; table 2) at 10 days post lesion and nearly reached the control values at 21 days post lesion (table 2). In contrast, the TA muscles from HSP70 mice did not have significant alterations in fiber CSA (table 2) in all time points evaluated.

Histological cross-sections of soleus and TA muscles from non-lesioned mice and 1, 10 and 21 days after cryolesion were stained with toluidine blue and microscopically analyzed. In line with a normal tissue structure, the intact control muscles from wild type and HSP70 mice exhibited fibers with polygonal shape and peripheral nuclei (figs. 2A, 2B, 3A, 3B).

At 1 day post injury, the cryolesion induced significant damage in soleus and TA muscles from wild type mice, observed by presence of hypercontracted fibers, inflammatory cell infiltration and empty spaces between the cells which clearly indicate myonecrosis, tissue disruption and edema (figs. 2C, 3C). At the same time point, the muscles from HSP70 mice had minimized signals of lesion, i.e. fewer hypercontracted fibers, less empty spaces between the cells and less inflammatory process (figs. 2D, 3D). Also the percentages of necrosed myofibers in soleus and TA muscles from wild type mice at 1-day post lesion were significantly higher than those from HSP70 mice (table 3).
Soleus and TA muscles were treated with radicicol immediately following cryolesion and analysed 1 day later. These muscles, similar to the HSP70 mice, had significantly minimized signals of lesion and fewer incidences of necrosed myofibers when compared to the cryolesioned-only muscles (Fig. 4, table 3). The muscles only injected with radicicol or the vehicle ethanol did not have morphological alterations (Fig 4).

The soleus and TA muscles examined at 10 days of regeneration from both wild type and HSP70 mice had cells in different stages of regeneration with presence of inflammatory cell infiltration, basophilic regenerating cells and centrally nucleated cells of various sizes (figs. 2E, 2F, 3E, 3F). However, the muscles from wild type had more inflammatory process and neighboring cells with smaller cross-section area when compared to those of HSP70 mice, which is easily observed in the TA muscle (Fig. 3E).

At 21 days after cryolesion, the soleus and TA muscles from wild type and HSP70 mice were mostly regenerated, as highlighted by the presence of classical signals of regeneration (Karpati et al. 1988), i.e. cells with centralized nuclei and split fibers (Figs. 2G, 2H, 3G, 3H).

GPx enzymatic activity was determined in homogenates obtained from TA muscles of wild type and HSP70 mice in order to assess the antioxidant defense against free radicals produced in tissue damage. At 1 day after cryolesion, the GPx activity similarly increased significantly in TA muscles from both wild type and HSP70 transgenic mice (2.4 and 1.7 fold, respectively, fig. 5). GPx activity remained elevated in the muscles from both wild type and HSP70 mice up to 21 days after cryolesion (in 10 days: 2.1 and 2.3 fold, respectively and in 21 days: 2.1 and 1.7 fold, respectively; fig. 5).
In addition, the GPx activity in TA muscles from HSP70 mice was significantly higher than in those from wild type mice 10 days post injury (1.4 fold, fig. 5).

As there was no difference between soleus and TA muscles regarding general structural morphology of the injury response in HSP70 transgenic mice we decided to carry on a more detailed evaluation of skeletal muscle protection against lesion and regenerative response in TA only of HSP70 mice.

TA muscle cross-sections were immunostained with antibody against NCAM to detect and count the SCs at 1, 10 and 21 days after cryolesion. As expected, the amount of NCAM positive SCs in normal intact muscles from wild type mice was low (TA: ~0.3%, Fig. 6) (Charge and Rudnicki 2004). The HSP70 mice also had a basal number of SCs similar to that of wild type mice (Fig. 6). At 1 and 10 days after cryolesion, the number of SCs was increased in TA (7.6 and 4.3 fold, respectively) muscles from wild type mice (Figs. 6). Although the muscles from HSP70 mice followed the same trend as muscles from wild type mice in the increase in SCs at 1 and 10 days post lesion, this amount was significant only at 10 days (5 fold, fig. 6). At 21 days of regeneration it is noticeable that the number of SCs returns to the control values in TA muscles (Fig. 6).

The MHCd/n immunostaining was performed in order to detect the presence of differentiating myofibers in regeneration (Davis et al. 1991). The expression of MHCd/n was detected only at 10 days of regeneration, in which TA muscles from HSP70 mice had fewer MHCd/n positive cells compared to wild type mice (26.6±4.7% vs 42.2±3%; respectively, Fig. 7).
DISCUSSION

The results of the present work demonstrate the overexpression of HSP70 protein in transgenic mice leads to considerable alterations in the response of the soleus and TA muscles to cryolesion compared to control mice muscles.

Initially, we confirmed that the transgenic mice used in the present study were in fact overexpressing HSP70 (fig. 1a & b). Furthermore, we show a slight induction of HSP70 in cryolesioned skeletal muscle at 1 day post-lesion and a substantial induction at 10 days post-lesion. Also, treatment of wild type mouse skeletal muscle with radicicol causes a marked increase in expression of HSP70 at 1 day after radicicol administration (fig.1c). Therefore, although cryolesion does result in a slow increase in HSP70, administration of radicicol results in a rapid increase in HSP70 expression. In addition, we show that in the basal state (no lesion), the body and muscle weights and fiber CSA of both soleus and TA muscles (representative of slow and fast twitch muscles, respectively) were significantly lower in HSP70 mice when compared to wild type mice (table 1). These results are in line with a previous study (McArdle et al. 2004) which showed that overexpressing HSP70 transgenic mice had reduced body weight by ~10%, muscle weight by ~20% and fiber CSA by ~16% compared with body and muscle weights and fiber CSA of wild type mice. On the other hand, the fiber type proportions were unaltered in both soleus and TA muscles from HSP70 mice when compared to controls, indicating that the phenotype of the skeletal muscle fiber types is unaffected by overexpression of HSP70.

Cryolesion is a model that provides an excellent opportunity to assess both the protective nature of the skeletal muscle against injury and as well as its regenerative
capacity. This model (Wernig and Irintchev 1995; Wernig et al. 1995) is well recognized to induce necrosis in a well-delineated area of skeletal muscles and subsequently, regeneration (Irintchev et al. 2002; Miyabara et al. 2005). In the present study, soleus and TA muscle cross-sections were evaluated at 1, 10 and 21 days after cryolesion. These time points were chosen as they represent well the structurally and functionally distinct phases of skeletal muscle adaptations to injury, including intense necrosis and edema; evident regeneration; and restoration of the structural features of skeletal muscle normal morphology (Irintchev et al. 2002; Miyabara et al. 2005).

The molecular mechanisms underlying tissue protection against a severe injury such as cryolesion is currently unclear. It is well known that sarcolemma disruption followed by a rise in intracellular Ca\(^{++}\) concentration and proteolysis are key events for skeletal muscle fiber degeneration (Charge and Rudnicki 2004) and the expression of heat-shock genes occurs in response to any stress that produces denatured proteins, including a Ca\(^{++}\)-mediated injury (Kuhlmann et al. 1997). Therefore it is likely that denatured sarcolemma proteins initiate a heat-shock response after cryolesion, possibly by binding HSP90, which results in the dissociation of HSP90 from heat shock factor proteins (HSFs) (Ananthan et al. 1986). Under basal conditions HSP90 binds to and suppresses HSF activation (Zou et al. 1998). The dissociation of HSP90 from HSFs can lead to the phosphorylation and subsequent activation of HSFs. The activated HSFs then form trimers (Wu 1995) that bind to the promoter of the HSP genes to stimulate transcription (Morimoto et al. 1997). Consequently, the levels of HSP70 mRNA and protein are increased in stressed cells. Once synthesized the HSP70 binds to denatured proteins and attempts to restore their tertiary structure and enzymatic activity in a cycle
driven by ATP hydrolysis (Gebauer et al. 1997). In addition, cryolesion induced HSPs could be involved in the inflammatory process during skeletal muscle injury, as it has been shown that they participate in cytokine signaling and cytokine gene expression and enhance antigen presentation to T lymphocytes (Moseley 1998).

At 1 day after cryolesion, the soleus and TA muscles from HSP70 mice clearly have minimized signals of lesion, when compared to the muscles from wild type mice, which shows that the overexpression of HSP70 attenuates skeletal muscle damage induced by cryolesion (figs. 2, 3). Skeletal muscle protection in HSP70 mice is indicated by sparing injured TA muscle from weight loss (table 2). Accordingly, a dramatic decrease in necrosis at 1 day after injury was observed in soleus and TA muscles from HSP70 mice as compared to wild type mice (table 3). In addition, at 10 day post lesion, the decrease in fiber CSA is attenuated in the soleus and TA muscles from HSP70 animals relative to wild type animals (table 2). These results are in line with a previous study (McArdle et al. 2004) which showed that the overexpression of HSP70 in transgenic adult and old mice is able to significantly minimize the force deficit in muscles submitted to a lengthening contraction damage when compared to muscles from wild type mice, suggesting that HSP70 also contributes to functional preservation of skeletal muscle in response to damage. Nonetheless, future studies, particularly at the single skeletal muscle fiber level, are required to investigate if the HSP70 protects/preserves contractile/metabolic functions following injury. At that point, although limited histological analysis had been performed, it was completely unclear how the regenerative response would be influenced by HSP70 overexpression. In the present study it is clear
that the skeletal muscle regenerative process is less recruited. Thus, overexpression of HSP70 appears to provide protection rather than inducing improved regeneration.

To assess the relative contributions of the antioxidant defense system we examined GPx activity after cryolesion. GPx is an important enzyme involved in the glutathione-glutathione disulfide cycle to remove types of reactive oxygen species (ROS), more specifically the H$_2$O$_2$ and organic hydroperoxides, which are produced in the cytosol and mitochondria of damaged cells (Powers and Lennon 1999). We observed that GPx activity is elevated 1 day after cryolesion and maintained over the experimental procedure (up to 21 days) in both wild type and HSP70 mice (fig. 5) and further that muscles from HSP70 mice had significantly higher GPx activity at 10 days after cryolesion compared to wild type muscles. This suggests that overexpression of HSP70 exerts a positive effect (increased expression/activity) on GPx, which would protect the skeletal muscle against injury.

The results obtained in the present study prompted us to investigate the effect of a pharmacological HSP inducer, radicicol, on injured skeletal muscles. In line with the results from the HSP70 mice, radicicol, which quickly increases the expression of HSP70, significantly reduced the cryolesion-induced damage at 1 day (fig. 4, table 3).

One interpretation on the data above is that elevated levels of HSPs might have a sparing effect on skeletal muscle from injury, without having significant effect on skeletal muscle regeneration per se. This, in fact, would be in line with the molecular effects of HSPs, i.e. restoration of tertiary structure of denatured proteins, maintaining cell structure and function (Gebauer et al. 1997). On the other hand, HSPs may modulate the skeletal muscle regeneration process after injury. Therefore, we aimed to investigate
the regenerative capacity of cryolesioned skeletal muscles from HSP70 mice. In order to better assess the effect of HSP70 in the regenerating process, we examined the expression of NCAM and MHCd/n.

It is noteworthy that no differences in histological features, i.e. analysis of muscles stained with toluidine blue/acid phosphatase and fiber CSA, were detected between soleus and TA muscles from HSP70 mice, demonstrating that the important role of HSP70 in skeletal muscle takes place independently of fiber type. Therefore in the subsequent analysis (focused on skeletal muscle regenerative capacity, i.e. SC number and MHCd/n immunodetection) we have evaluated only TA muscle.

NCAM is a SC marker commonly used to study skeletal muscle plasticity (Dedkov et al. 2001; Dedkov et al. 2003). We found elevated amount of NCAM positive SCs is raised at 1 and 10 days after cryolesion in TA muscle from wild type mice (fig. 6). Indeed, a previous work has demonstrated that the number of muscle cells expressing NCAM is increased at both 2 and 11 days of regeneration after a denervation model (Muller-Felber et al. 1993). Furthermore, it has been demonstrated that SCs become activated within 6 h of injury and in response to locally released growth factors from injured myofibers and macrophages, and SCs proliferate extensively within 2-3 days of cardiotoxin-induced injury (Hawke and Garry 2001). Our results clearly show a lower number of SCs in skeletal muscles of HSP70 mice 1 day post injury in TA muscle (Fig 6). This suggests as skeletal muscles from HSP70 mice are partially spared from the cryolesion-induced damage, they require less activation of SCs to repair the skeletal muscle profile. This interpretation leads to the idea that HSP70 mice are protected against injury, with a normal regenerative capacity.
To further examine the regenerative response of HSP70 cryolesioned skeletal muscle MHCd/n immunoexpression was examined. MHCd/n is known to be strongly up-regulated after skeletal muscle injury in adult animals (Jerkovic et al. 1997; Duguez et al. 2003). In our study, the MHCd/n expression is detected only at 10 days after cryolesion in TA (fig. 7), in agreement with a previous study that demonstrated MHCd/n gene induction at 10 days post-cryolesion (Miyabara et al. 2005). Also, it has been shown that MHCd/n expression is progressively increased to peak on day 7 post-injury and is no longer detected on day 14 (Duguez et al. 2003). The 10 day analysis of MHCd/n immunoexpression in our study reveals that the muscles from HSP70 mice have comparatively fewer regenerating cells contributing to the idea that the decreased damage associated with HSP70 expression subsequently necessitates fewer regenerating cells.

Although signals of regeneration are quite diminished in injured skeletal muscles of HSP70 mice, at the present time, one cannot ascertain whether HSPs modulate the regenerating capacity in skeletal muscle. It is important to highlight that wild type and HSP70 muscles had different degrees of necrosis after injury in our experimental model, therefore it is difficult to determine whether the regenerative response after injury was in fact changed. Future studies utilizing injuries in progressive intensities in wild type and HSP70 animals should be performed in order to compare the regenerative response under a similar damage status.

In summary, these results suggest that the expression of HSP70, whether by transgenesis or pharmacological induction, strongly attenuates the skeletal muscle injury after cryolesion and therefore, protects the skeletal muscle in the early stages after damage. In addition, this study is the first to provide histological evidence for the role of
radicicol as a potential therapeutic agent to be used in the treatment of skeletal muscle injury. Nonetheless, functional studies need to be performed to deepen our knowledge of HSPs as tissue protectors and also to highlight potential benefits of radicicol treatment.

GRANTS

We thank FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil, grant 02/03195-2 to EHM) for funding this study. This work was also supported by NIH HL61339 & HL67971 (to RM) and an award from the American Heart Association (to JLM).
REFERENCES


34. **Salvini TF, Morini CC, Selistre de Araujo HS and Ownby CL.** Long-term regeneration of fast and slow murine skeletal muscles after induced injury by ACL myotoxin isolated from Agkistrodon contortrix laticinctus (broad-banded copperhead) venom. *Anat Rec* 254: 521-533, 1999.


Table 1. Characterization of body (g) and muscle (mg) weights, fiber type composition (%) and cross-section area (CSA; µm²) of immunohistochemically classified fiber types of soleus and TA muscles from wild type (Wt) and transgenic HSP70 mice.

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>HSP70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>28.5±2.3</td>
<td>22±2.5  *</td>
</tr>
<tr>
<td>Muscle weight (mg)</td>
<td>6.8±1.3</td>
<td>41.5±2.4</td>
</tr>
<tr>
<td>Type I (%)</td>
<td>36.7±4.6</td>
<td>---</td>
</tr>
<tr>
<td>Type II (%)</td>
<td>63.3±4.6</td>
<td>100</td>
</tr>
<tr>
<td>Type I, CSA (µm²)</td>
<td>198±1.5</td>
<td>---</td>
</tr>
<tr>
<td>Type II, CSA (µm²)</td>
<td>231±1.5</td>
<td>242±1.3</td>
</tr>
<tr>
<td>TA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD; n=6-7. An unpaired t-student test was applied to test differences between 2 groups (Wt and HSP70). Significantly different from matched-Wt: *p<0.05.
Table 2. Muscle weight (MW; mg) and fiber cross-section area (CSA; μm²) of soleus and TA muscles from wild type (Wt) and transgenic HSP70 mice in different times of regeneration.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Soleus Wt (MW) (fold)</th>
<th>HSP70 Wt (MW) (fold)</th>
<th>TA Wt (MW) (fold)</th>
<th>HSP70 TA (MW) (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.8±1.3 (1)</td>
<td>5±0.4 * (1)</td>
<td>41.5±2.4</td>
<td>33.4±2.5 * (1)</td>
</tr>
<tr>
<td>1 day post lesion</td>
<td>12±4.2 a (1.8)</td>
<td>8±1* b (1.6)</td>
<td>52.1±4.4 a</td>
<td>41±3.4 * (1.2)</td>
</tr>
<tr>
<td>10 days post lesion</td>
<td>8±0.7 (1.2)</td>
<td>5.4±1.1 * (1.1)</td>
<td>34.6±3.6</td>
<td>24.3±1.8 * b (0.7)</td>
</tr>
<tr>
<td>21 days post lesion</td>
<td>8.1±0.9 (1.2)</td>
<td>6.5±1.5</td>
<td>39.9±5.6</td>
<td>39.7±6.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>Wt (CSA) (fold)</th>
<th>HSP70 Wt (CSA) (fold)</th>
<th>TA Wt (CSA) (fold)</th>
<th>HSP70 TA (CSA) (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>157±1.6 (1)</td>
<td>120±1.5 * (1)</td>
<td>178±14</td>
<td>121±10 * (1)</td>
</tr>
<tr>
<td>10 days post lesion</td>
<td>71±0.5 a (0.45)</td>
<td>95±1.8 * b (0.8)</td>
<td>114±27 a</td>
<td>123±19</td>
</tr>
<tr>
<td>21 days post lesion</td>
<td>120±1.4 a (0.8)</td>
<td>115±1.8 * b (0.95)</td>
<td>209±44</td>
<td>172±36</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD and fold induction; n=5-7. A two-way ANOVA followed by the Tukey’s procedure for multiple comparisons was applied to test differences among 3 (Control, 10 and 21 days post lesion) or 4 groups (Control, 1, 10 and 21 days post lesion) from Wt and HSP70 mice. a p<0.05 vs WT Control; b p<0.05 vs HSP70 Control and * p<0.05 vs matched-Wt.
**Table 3.** Incidence of necrosed myofibers of soleus and TA muscles at 1 day after cryolesion as assessed by acid-phosphatase reaction.

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>HSP70</th>
<th>Radicicol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Soleus (%)</strong></td>
<td>39.4±2.3</td>
<td>23.1±2.2 $^a$</td>
<td>24.9±5.4 $^a$</td>
</tr>
<tr>
<td><strong>TA (%)</strong></td>
<td>38.9±4.3</td>
<td>18.1±4.6 $^b$</td>
<td>17.5±3.6 $^b$</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD (n=3 for muscles from Wt: wild type mice; HSP70: transgenic HSP70 mice and Radicicol: wild type mice treated with radicicol). A one-way ANOVA followed by the Tukey’s procedure for multiple comparisons was applied to test differences among 3 groups (Wt, HSP70 and Radicicol) in soleus and TA muscles. Significantly different from Wt: $^a p<0.01$; $^b p<0.001$. 
Fig. 1: Western blot analysis of control and cryolesioned at 1 day post-lesion (a), control and cryolesioned at 10 days post-lesion (b) from the TA muscles of overexpressing HSP70 mice and wild type CB6F1 mice. Autoradiograph of a 8% SDS-PAGE with TA muscle protein (50ug per lane). Lane 1 & 2: total muscle protein extract from wild type CB6F1 mice, control (C) and lesioned (L) muscles respectively. Lane 3 & 4: total muscle protein extract from transgenic HSP70 mice, C and L muscles respectively. (c) Western blot analysis of protein extracts from TA muscles treated with radicicol (0.05mg/20g body weight) (1, 3) or treated with vehicle, ethanol (2, 4). Blot was reacted with antibody specific for HSP70 and subsequently with antibody to actin.
Fig. 2: Toluidine Blue stained soleus muscle cross-sections from wild type (A, C, E, G) and transgenic HSP70 overexpressing mice (B, D, F, H). A and B: control (nonlesioned) groups; C and D: muscles 1 day after cryolesion; E and F: 10 days after cryolesion; and G
and H: 21 days after cryolesion. Note that the nonlesioned groups (A and B) have normal and similar morphology. The muscle overexpressing HSP70 (D) had less signs of injury relative to the wild type muscle (C), i.e., less hypercontracted fibers (arrows; C, D), less inflammatory cell infiltration (asterisks; C, D) and less empty spaces between the fibers. At 10 days post lesion wild type and overexpressing HSP70 muscles have regenerating cells with centralized nuclei (big arrows; E, F), although the wild type soleus have increased inflammatory process (asterisk, E) and empty spaces when compared to transgenic soleus sections (asterisk, F). At 21 days after cryolesion, both wild type and transgenic HSP70 muscles had clear signals of regeneration, i.e. fibers with centralized nuclei (big arrows; G, H) and split fibers (small arrows; G, H); and minimized inflammation and wider spaces between fibers (asterisks; G, H), which were still greater in wild type muscles (asterisk, G). Bar: 50 μm.
**Fig. 3:** Toluidine Blue stained TA muscle cross-sections from wild type (A, C, E, G) and transgenic HSP70 overexpressing mice (B, D, F, H). A and B: control groups; C and D: muscles 1 day after cryolesion; E and F: muscles 10 days after cryolesion; G and H: muscles 21 days after cryolesion. Note that the control groups (A and B) have normal morphology. The muscle overexpressing HSP70 (D) had fewer indicators of damage relative to the wild type muscle (C), i.e., fewer hypercontracted fibers (arrows; C, D), less inflammatory cell infiltration (asterisks; C,D) and less empty spaces between the fibers. Ten days post lesion wild type and HSP70 overexpressing muscles display cells with centralized nuclei (big arrows; E, F), although wild type muscle typically had more inflammatory process (asterisk, E) and neighboring basophilic regenerating cells or centrally nucleated cells compared to transgenic muscle (asterisk, F). At 21 days after cryolesion, both wild type and transgenic HSP70 muscles had clear signals of regeneration: fibers with centralized nuclei (big arrows; G, H) and split fibers (small arrows; G, H). Bar: 50 μm.
Fig. 4: Toluidine Blue stained cross-sections of soleus (A, C, E) and TA (B, D, F) muscles from wild type mice. Muscles injected with the vehicle ethanol had normal structural morphology (A, B). The soleus and TA muscles cryolesioned and analyzed after 1 day had empty spaces among the muscle fibers which results from disruption and edema, inflammatory cell infiltration (asterisks; C, D) and hypercontracted fiber (arrow, D). The muscles injected with radicicol after cryolesion had minimized signals of lesion, i.e. less inflammatory cells (asterisks; E, F), less empty spaces among the cells and still
some hypercontracted fibers (arrow, F) when compared to the muscles only cryolesioned. Bar= 50 μm.
Fig. 5: Glutathione peroxidase (GPx) activity from TA muscles of control and 1, 10 and 21 days after cryolesion in wild type (Wt) and HSP70 (HSP70) mice. Data are expressed as mean ± SE, n=4; *p<0.05 vs. Wt and HSP70 controls, #p<0.05 vs. Wt at the same time.
**Fig. 6:** a: Confocal images of TA muscle cross-sections immunostained for the satellite cell marker, NCAM, from wild type (A, C, E, G) and transgenic HSP70 overexpressing mice (B, D, F, H). A and B: control groups; C and D: 1 day post lesion; E and F: 10 days post lesion. G and H: 21 days post lesion. Arrows point to NCAM. Bar= 2 μm. b: Comparison of percent NCAM positive cells in control and 1, 10 and 21 days after cryolesion in TA muscles from wild type (Wt) and HSP70 (HSP70) mice. Data are expressed as mean ± SE; n= 3 whole cross-sections; *p<0.05 vs. Wt Control; &p<0.05 vs. HSP70 Control; #p<0.05 vs. HSP70 at the same time.
Fig. 7: a: Light microscopy images of developmental/neonatal myosin heavy chain (MHCd/n) immunostaining of regenerating muscles in representative cross-sections. A and B: are from TA muscles of wild type and HSP70 mice, respectively, 10 days after cryolesion and counterstained with hematoxylin. Arrows point to the cells positive for MHCd/n and asterisks are inside the normal intact cells. Note that the TA from wild type mice (A) display higher expression of MHCd/n compared to HSP70 mice (B). Bar: 50 μm. b: Comparison of MHCd/n positive cells from wild type (Wt) and HSP70 (HSP70)
mice as percent of whole TA cross-sectional area, analyzed 10 days after cryolesion. Data are expressed as mean ± SD; n= 3 whole cross-sections; *p<0.05 vs. Wt Cryo.