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Modulation Of Hsp25 And Tnf-alpha During The Early Stages Of Functional Overload Of A Rat Slow And Fast Muscle

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Modulation of HSP25 and TNF-α during the early stages of functional overload of a rat slow and fast muscle

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Huey KA, McCall GE, Zhong H, Roy RR. Modulation of HSP25 and TNF-α during the early stages of functional overload of a rat slow and fast muscle. *J Appl Physiol* 102: 2307–2314, 2007. First published March 22, 2007; doi:10.1152/japplphysiol.00021.2007.—Early events in response to abrupt increases in activation and loading with muscle functional overload (FO) are associated with increased damage and inflammation. Heat shock protein 25 (HSP25) may protect against these stressors, and its expression can be regulated by muscle loading and activation. The purpose of this study was to investigate the responses of HSP25, phosphorylated HSP25 (pHSP25), and tumor necrosis factor-α (TNF-α) during FO of the slow soleus and fast plantaris. We compared the HSP25 mRNA, HSP25 protein, pHSP25, and TNF-α responses in the soleus and plantaris after 0.5, 1, 2, 3, and 7 days of FO. HSP25 and pHSP25 were quantified in soluble and insoluble fractions. HSP25 mRNA increased immediately in both muscles and decreased with continued FO. However, HSP25 mRNA levels were consistently higher in the muscles of FO than control rats. In the soluble fraction, HSP25 increased in the plantaris after 2–7 days of FO with the greatest response at 3 and 7 days. The pHSP25 response to FO was greater in the plantaris than soleus at all points in the soluble fraction and at 0.5 days in the insoluble fraction. TNF-α levels in the plantaris, but not soleus, were higher than control at 0.5–2 days of FO. This may have contributed to the greater FO response in pHSP25 in the plantaris than soleus as TNF-α increased pHSP25 in C_2C_{12} myotubes. These results suggest that the initial responses of pHSP25 and TNF-α to mechanical stress and inflammation associated with FO are greater in a fast than slow extensor muscle.

heat shock protein 25; phosphorylation; tumor necrosis factor-alpha; skeletal muscle

HEAT SHOCK PROTEINS (HSP) were initially discovered and named based on their rapid induction in response to elevated temperatures. However, ongoing research has clearly demonstrated that HSPs respond to a wide variety of physical and chemical stresses (6, 56), and growing evidence supports their important role in maintaining skeletal muscle structure and function (35). Whereas one of the larger HSPs, HSP70, has been studied more extensively in skeletal muscle, limited data exist regarding the role of small HSPs, such as HSP25 (homologous to human HSP27) in skeletal muscle function and integrity. However, a role for HSP25 in skeletal muscle is underscored by evidence suggesting its ability to protect muscle cells from stressors such as atrophy associated with decreased use (33, 48), oxidative damage (10), and muscle mechanical damage (21, 22, 28). Furthermore, reduced HSP25 expression has been implicated in muscle diseases such as muscular dystrophy (46). Although the functions of HSP25 in chaperoning unfolded proteins, stabilizing the cytoskeleton, conferring resistance to oxidative stress and inflammation, and inhibiting apoptosis (6) are documented in other cell types such as fibroblasts and smooth muscle cells, its specific functions in skeletal muscle are less clear. Importantly, factors that regulate the phosphorylation state of HSP25 (pHSP25) in skeletal muscle are poorly understood. Recent studies are only beginning to isolate the factors that impact HSP25 phosphorylation in muscle (16, 20), and more studies are necessary to provide the groundwork for future studies elucidating how phosphorylation impacts the functional properties of HSP25 in muscle.

The abrupt and chronic increases in muscle loading and activation induced by synergist removal (2, 3, 12, 25) provide a relevant model to investigate initial factors that contribute to changes in the expression and phosphorylation of HSP25. Previously, our laboratory reported increases in HSP25 and pHSP25 after 3 or 7 days of functional overload (FO) (15); however, responses critical for the remodeling of the muscle likely begin soon after the onset of overload. For example, early changes include elevated expression of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) (53) and the rapid phosphorylation of the mitogen-activated protein (MAP) kinases within 24 h of muscle overload (4). MAP kinase activation is a good indicator of immediate increases in muscle loading and activation (29), changes that likely contribute to muscle damage (5, 50) in the overloaded muscles. Because the immediate translocation of HSP25 and/or pHSP25 to the insoluble fraction has been shown to stabilize cytoskeletal elements and help maintain muscle structure after damaging contractions (22), it is likely that alterations in HSP25 and pHSP25 are among the earliest responses to FO.

During the initial stages of FO, increases in muscle weight are generally indicative of inflammation (2) rather than the increases in muscle protein levels (1). The expression of TNF-α is significantly elevated in overloaded muscle (53), and HSP25 has been shown to protect cells from TNF-α-induced damage (30, 40, 44). Importantly, both TNF-α and muscle damage are associated with increased HSP25 phosphorylation. TNF-α-dependent phosphorylation of HSP25 has been shown in fibroblasts via activation of p38 MAP kinase and MAP kinase-activated protein kinase 2 (9, 39, 44). Furthermore, following damaging muscle contractions, increases in HSP25 in the insoluble fraction, where it may stabilize or repair cytoskeletal elements, is associated with increased HSP25 phosphorylation (22). Taken together with data in nonmuscle

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cells (14, 18, 23, 24), the ability of HSP25 to stabilize cytoskeletal elements is likely phosphorylation dependent. Thus muscle damage induced by the abrupt increases in muscle loading associated with FO (5, 50) may be attenuated by increases in pHSP25.

To date, no studies have investigated how rapidly HSP25 expression is induced in response to the initial stress of FO and the associated inflammatory response. Furthermore, the time course of changes in the phosphorylation state of HSP25 under these conditions and whether TNF-α is involved in this response are unknown. Moreover, a comparison of the adaptations in HSP25 expression and phosphorylation between a predominately slow and a predominantly fast muscle will provide unique insight into muscle-specific factors that regulate the HSP25 response. Therefore, the primary purpose of the present study was to test the hypothesis that rapid increases in HSP25 phosphorylation and/or HSP25 expression occur in response to overload in a muscle-specific fashion. We compared HSP25 mRNA, HSP25 protein, and pHSP25 in the rat soleus and plantaris muscles, plantar flexors with different myosin heavy chain (MHC) profiles and mechanical properties, after 0.5, 1, 2, 3, and 7 days of FO. Because the soleus is a predominantly slow, highly oxidative, and tonically active muscle, whereas the plantaris is a predominantly fast, more glycolytic, and more phasically active muscle, we hypothesized that the HSP25 response (fold change from control baseline values within each muscle) in the soleus would be lower than the plantaris due to the greater relative changes in loading and activation induced by FO in the fast than the slow muscle. HSP25 and pHSP25 levels were quantified in both the soluble and insoluble fractions to test the hypothesis that the increased activation and loading imposed on the muscles (and probable muscle damage) increases HSP25 and/or pHSP25 levels in the insoluble fraction where it may help stabilize cytoskeletal elements (22). We also investigated changes in TNF-α as a possible mechanism contributing to increases in HSP25 phosphorylation in skeletal muscle both in vivo, in the overloaded soleus and plantaris, and in vitro, in C_2C_{12} myotubes. We hypothesized that TNF-α increases HSP25 phosphorylation in isolated skeletal muscle cells and that muscle-specific differences in TNF-α levels are associated with differences in the pHSP25 responses.

MATERIALS AND METHODS

Experimental design. This study was approved by the Animal Use Committee at the University of California, Los Angeles, and it followed the American Physiological Society animal care guidelines. Adult, female Sprague-Dawley rats (initial mean body weight of 228 ± 1 g) were assigned randomly to a *day 0* or *day 7* control group (Con-0d and Con-7d, respectively) and one of the following FO groups: *1*) 0.5-day FO (FO-0.5d), *2*) 1-day FO (FO-1d); *3*) 2-day FO (FO-2d), *4*) 3-day FO (FO-3d), or *5*) 7-day FO (FO-7d) (n = 5/group for each time point). The overload period was initiated when the animals began moving around the cages. There were no significant differences in initial body weight among the groups. All surgical procedures were performed under aseptic conditions with the rats deeply anesthetized (100 mg ketamine and 5 mg acepromazine/kg body wt). Overload of the plantaris and soleus muscles was performed bilaterally via removal of their major synergistic muscle, the gastrocnemius, as described in detail previously (3). Based on our previous (15) findings, sham operations were not performed on the Con groups. We compared HSP25 expression and phosphorylation between control and 1-day (n = 3) or 3-day (n = 4) sham-operated animals and found no differences in either the soleus or plantaris values. Comparisons between control and 3-day sham-operated soleus and plantaris were reported previously (15). Values in the sham-operated soleus were normalized to control (1.0) and were 0.94 ± 0.02 and 0.98 ± 0.02 for HSP25 and pHSP25, respectively, after 1 day. Normalized values in the sham-operated plantaris were 0.99 ± 0.02 and 1.05 ± 0.04 for HSP25 and pHSP25, respectively, after 1 day. Furthermore, there were no significant changes in HSP25 mRNA in either the plantaris or soleus after 1 or 3 days of sham surgery. All of these data indicate that the results reported in the present study are attributable to the muscle overload and not the surgical procedures. At the predetermined time points, the rats were euthanized with an overdose of pentobarbital sodium (100 mg/kg body wt), and the plantaris and soleus muscles were removed bilaterally, quickly trimmed of excess fat and connective tissue, wet weighed, frozen in liquid nitrogen, and stored at −80°C until subsequent analysis. The muscles from a single leg of each animal were analyzed for both mRNA and protein. The midbelly of each muscle was used for analysis.

mRNA analyses. Muscle samples (50–60 mg) were homogenized in 1 ml of TRI Reagent (Molecular Research Center, Cincinnati, OH), and RNA was extracted according to the manufacturer's protocol. RNA concentration was determined with a spectrophotometer and stored at −80°C for subsequent RT-PCR.

Quantitative real-time RT-PCR was used to determine the expression of HSP25 mRNA relative to β-actin. One microgram of total RNA from each muscle sample was reverse transcribed using the Stratascript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

For gene expression analysis, 1 μl of the RT reaction was added to a reaction mix containing 1× Full-velocity SYBRgreen (Stratagene) and 150 nM of the HSP25 or β-actin primer pairs. Amplification and quantitation of mRNA were performed on a Stratagene Mx3000P with the following parameters: a denaturing step at 95°C for 5 min, followed by 10 s at 95°C and 30 s at 60°C for 40 cycles. The primer sequences were as follows for HSP25: forward 5′-TGGCTACATCTCTCGGTGCT-3′, reverse 5′-ATGGTGATCTCCGCTGATTG-3′ and β-actin: forward 5′-TGGAGAAGATTTGGCACCA-3μ, reverse 5′-CCAGAGGCATACAGGGACAA-3′ (Operon, Huntsville, AL) and generated fragments of 150 and 193 bp, respectively. All primer pairs produced a dilution curve of cDNA with a slope of 100 ± 10% "efficiency" where 100% = $\Delta 3C_T$/log cDNA input (C_T is the threshold PCR cycle at which fluorescence is detected above baseline). Fluorescence measurements were taken at the end of each cycle (product extension period). After amplification, a melting curve analysis was performed to verify amplification product specificity. Briefly, amplification products were denatured at 95°C for 1 min, and then they were quickly cooled to 55°C for 30 s. This was followed by a ramp up to a final temperature of 95°C while continuously measuring fluorescence. The derivative of the melting curve fluorescence was plotted vs. temperature, thus yielding melting peaks indicative of products generated during amplification. The lack of primer-dimer complexes and the presence of an appropriate single peak in the resultant melting curve were indicators of correct amplification products. In initial experiments, PCR products were separated on 2% agarose gels, stained with ethidium bromide, and photographed under UV light to validate that the PCR products were the appropriate size and that no artifact bands were present. Additional controls included the elimination of Stratascript RT enzyme during cDNA synthesis (demonstrating the absence of DNA contamination) and the elimination of cDNA during PCR to demonstrate no contaminants in the PCR Master Mix. All PCR reactions were performed in duplicate for each RT product. The relative expression level of HSP25 was normalized by subtracting the corresponding β-actin threshold cycle (C_T) values and using the $\Delta\Delta C_T$ comparative method (47).

Protein analyses. Plantaris or soleus (n = 5/time point) muscles from Con-0d, Con-7d, FO-0.5d, FO-1d, FO-2d, FO-3d or FO-7d

animals were homogenized in 10 volumes of an ice-cold buffer containing 50 mM Tris·HCl (pH 7.8), 2 mM potassium phosphate, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 3 mM benzamidine, 1 mM sodium orthovanadate, 10 mM leupeptin, 5 mg/ml aprotinin, and 1 mM 4-[(2-aminoethyl) benzenesulfonyl fluoride] using a motor-driven glass pestle. The homogenate was immediately centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant was removed as the detergent-soluble fraction. The detergent-insoluble fraction was obtained by resuspending the pellet in 10 mM Tris (pH 6.8), 2% SDS, and 2.5% β-mercaptoethanol and homogenizing with a motor-driven glass pestle. The remaining insoluble fraction was removed by centrifugation. Protein concentrations were determined using the Bio-Rad Protein Assay with bovine serum albumin (BSA) used for the standard curve. The samples were saved immediately in aliquots at −80°C for subsequent use in immunoblotting.

Western blot analysis was used to determine HSP25 and pHSP25 protein levels in Con-0d, Con-7d, FO-0.5d, FO-1d, FO-2d, FO-3d, or FO-7d soleus and plantaris muscles. Thirty micrograms of detergent-soluble or -insoluble proteins were boiled (5 min at 95°C) and separated by one-dimensional SDS-PAGE (12.5%) and transferred to nitrocellulose membranes (pore size 45 μm). After protein transfer, the membranes were blocked for 1 h in Tris-buffered saline (TBS)-5% milk. Following blocking, the membranes were incubated overnight at 4°C with an anti-HSP25 (1:5,000) or anti pHSP25 (Ser82) (1:1,000) antibody in TBS-2.5% BSA. The HSP antibodies were purchased from Stressgen (Ann Arbor, MI). Blots were washed three times in TBS-0.1% Tween and incubated with anti-rabbit secondary antibodies (Amersham Biosciences, Piscataway, NJ) at 1:5,000 for 1 h at room temperature. Blots then were washed in TBS-0.1% Tween, and the bound antibodies were detected with enhanced chemiluminescence (Amersham Biosciences). Blots were first probed for pHSP25 and subsequently stripped and reprobed for HSP25 to normalize pHSP25 to total HSP25.

Quantification of the bands was performed using ImageQuant Analysis software. All the necessary controls for Western blots were performed to ensure antibody specificity. To ensure uniformity, only bands within the same blot were used to compare the relative amounts of each protein among groups. The intensity of phosphorylated bands for HSP25 was first normalized to the intensity of the corresponding nonphosphorylated bands, and the ratio was normalized to control values.

TNF-α analyses. Soleus and plantaris muscles were homogenized in Tris buffer described above and the levels of TNF-α measured by ELISA (Endogen, IL) with detection limits of <1 pg/mg. These lower detection limits are sufficient to measure cytokine concentrations in muscle homogenates. Muscle TNF-α concentrations are expressed as picograms per milligram protein.

Cell culture and TNF-α experiments. Mouse C_2C_{12} myoblasts were maintained in DMEM supplemented with 10% fetal calf serum at 37°C and 5% CO_2. Differentiation and fusion were induced by switching cultures to DMEM supplemented with 2% horse serum (DM). Within 3 days in DM, cultures consistently fused into myotubes and all experiments were conducted after 4 days in DM. Initial dose-response experiments were performed with four TNF-α doses (0.1, 1, 10, or 100 ng/ml; Chemicon, CA), and the increase in pHSP25 was quantified. The optimal dose based on the pHSP25 response was determined to be 10 ng/ml, and this dose was used in all subsequent experiments. For experiments with the MAP kinase inhibitor, SB-203580, cells were pretreated with 1 or 10 μg 30 min before the addition of TNF-α. Experiments were performed by adding 10 ng/ml murine recombinant TNF-α for 15, 30, 60, or 120 min. Cell lysates were harvested in 100 μl of the Tris buffer and subjected to Western blot analysis as described above.

Statistical analyses. All data are presented as means ± SE. Because there were no differences in HSP25 mRNA, HSP25, or pHSP25 between Con-0d and Con-7d groups, these data were pooled, and a single Con value was calculated and compared with all other time points. Differences in muscle weights in the soleus or plantaris in Con-0d, Con-7d and after 0.5, 1, 2, 3, or 7 days of FO were determined using a one-way ANOVA. Two-way ANOVAs were used to assess main effects of days of FO (0.5–7 days), muscle (soleus vs. plantaris), or interactions between days of FO and muscle on HSP25 mRNA, HSP25 protein, pHSP25, or TNF-α in the soleus or plantaris muscles after 0 h (Con), 0.5, 1, 2, 3, or 7 days of FO. Differences in pHSP25 and HSP25 protein levels in C_2C_{12} cells after 0-, 30-, 60-, 90- or 120-min exposure to TNF-α were determined using a one-way ANOVA. If significant differences were found, the Bonferroni post hoc test was used to determine the source of the difference. Significant differences between initial and final body weights were determined with paired two-tailed *t*-tests. All analyses were performed with Graphpad Prism 4.0 with the significance level set at $P < 0.05$.

RESULTS

Changes in body and muscle weights. Initial mean body weights for the Con-0d, Con-7d, FO-0.5d, FO-1d, FO-2d, FO-3d, and FO-7d groups were 223 ± 2, 229 ± 6, 234 ± 2, 231 ± 3, 224 ± 4, 220 ± 1, and 233 ± 4 g, respectively. The terminal mean body weights varied among the groups as a result of significant increases from initial weights in the Con-7d and FO-7d groups compared with Con-0d (Table 1). Muscle weights were not increased above control values until 7 days of FO for either the plantaris or soleus. Mean absolute

Table 1. *Terminal body weight, absolute muscle weight, and relative muscle weight (to body weight) for control, 7-day control, 0.5-day functional overload, 1-day functional overload, 2-day functional overload, 3-day functional overload, and 7-day functional overload groups*

		Muscle Wet Weight, mg		Relative Muscle Wet Weight, mg/g	
	Body Weight, g	Soleus	Plantaris	Soleus	Plantaris
Con-0d	223±2	113±3	254±4	0.51±0.02	1.14±0.02
Con-7d	251±7§†	108±2	245±6	0.47±0.01	1.00±0.01
FO-0.5d	239±2	116±3	262±4	0.49±0.01	1.12±0.01
FO-1d	232±1*	126±2	263±2	0.55±0.01	1.14±0.01
FO-2d	225±3*	126±3	251±5	0.56±0.01	1.13±0.02
FO-3d	226±3*	127±7	265±8	0.58±0.03	1.20±0.03
FO-7d	246±2§†	150±7‡	308±7‡	0.64±0.03†	1.32±0.04‡

Values are means ± SE; $n = 5$/group. Con-0d, control; Con-7d, 7-day control; FO-0.5d, 0.5-day functional overload; FO-1d, 1-day functional overload; FO-2d, 2-day functional overload; FO-3d, 3-day functional overload; FO-7d, 7-day functional overload. *Significantly different from Con-7d and FO-7d, $P < 0.05$. †Significantly different from Con-0d, $P < 0.05$. ‡Significantly greater than all other time points, $P < 0.05$. §Significantly greater than Con-0d, Con-7d and FO-0.5d, ($P < 0.05$).

weights of the plantaris and soleus were 121 and 133% of Con-7d values, respectively, after 7 days of FO. Mean plantaris and soleus weights expressed relative to body weight were 115 and 121% of Con-7d values, respectively, after 7 days of FO.

Changes in HSP25 mRNA expression and protein content. There were significant main effects for days of FO ($P < 0.0001$) and for muscle examined ($P = 0.0001$), but there was no significant interaction effect for days of FO and muscle examined ($P = 0.172$) for the HSP25 mRNA response. HSP25 mRNA expression relative to β-actin was higher than Con at 0.5–2 days of FO in the plantaris and at 0.5 and 1 day of FO in the soleus (Fig. 1). The highest expression levels were observed at the earliest time point, with the values being ~7.5- and 5.5-fold higher than Con values after 0.5 days of FO in the plantaris and soleus, respectively. In general, the HSP25 mRNA expression decreased progressively over time such that the values for the plantaris at 3 and 7 days and those for the soleus at 2–7 days of FO were lower than at 0.5 days of FO. The values tended to be higher in the plantaris than soleus, but a significant difference was observed only after 2 days of FO.

The higher HSP25 mRNA levels in FO than Con rats were accompanied by elevated HSP25 protein levels in both the soleus and plantaris (Figs. 2 and 3). For HSP25 in both the soluble and insoluble fractions, there were significant main effects for days of FO ($P < 0.0001$ and $P < 0.0001$, respectively) and for muscle examined ($P = 0.0002$ and $P = 0.0248$, respectively) and a significant interaction effect for days of FO and muscle examined ($P = 0.0025$ and $P < 0.0001$, respectively). Compared with Con values, HSP25 levels in the soluble fraction were unaffected until 2 days of FO (Fig. 2*A*). From 2 to 7 days of FO, these values were significantly higher than Con in both muscles, with the response being significantly greater in the plantaris than soleus at 3 and 7 days. In addition, the HSP25 levels at 2–7 days of FO were significantly higher than at 0.5 and 1 day of FO for both muscles, except for the plantaris at 2 days of FO. In the insoluble fraction, HSP25 levels in the plantaris were significantly higher than Con at all time points, whereas in the soleus these values were significantly higher than Con only from *day 2* to *7* (Fig. 3*A*). At the early time points (0.5 and 1 day), the response was significantly greater in the plantaris than the soleus, whereas the response was significantly greater in the soleus than plantaris at the later time points ($P > 0.05$ at *day 7*). In addition, the HSP25 levels in the soleus were significantly higher at 2–7 days of FO than at 0.5 to 1 day of FO.

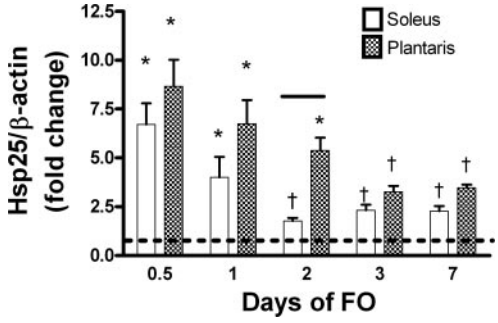

Fig. 1. Average fold changes in heat shock protein (HSP25) mRNA after 0.5, 1, 2, 3, and 7 days of functional overload (FO) in the plantaris and soleus muscles. The control value is set at 1.0 as indicated by the dashed line. Values are means ± SE. *Significantly different from control, $P < 0.05$. †Significantly different from 0.5 day, $P < 0.05$. Solid horizontal bar indicates a significant difference between the soleus and plantaris at $P < 0.05$.

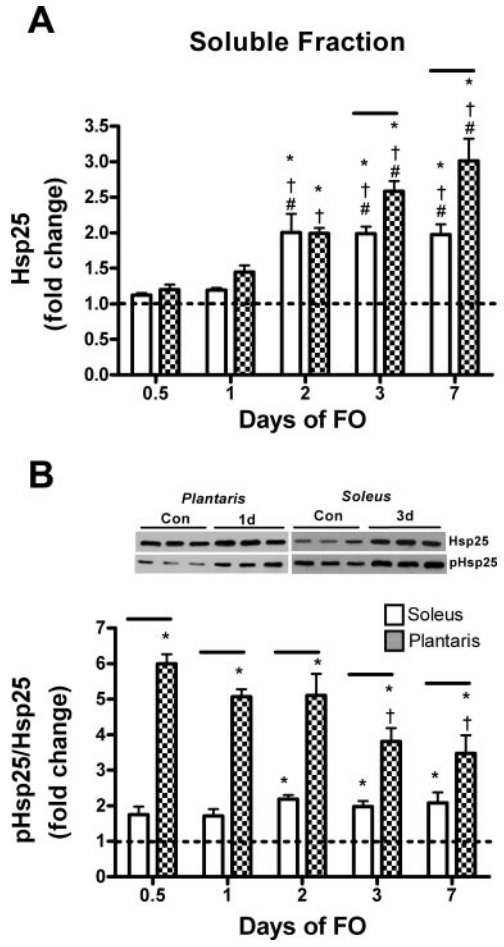

Fig. 2. Time course of the average relative changes (fold change from control) in HSP25 protein (*A*) and phosphorylated HSP25 (pHSP25; *B*) in the soluble fraction of the plantaris and soleus muscles after 0.5, 1, 2, 3, and 7 days of FO. In both graphs, the control value is set at 1.0 as indicated by the dashed line. Representative Western blots with 3-control (Con) and 3–1 day (1d) FO plantaris or 3-Con and 3–3 day(3d) FO soleus muscle samples are shown. The intensity of the phosphorylated bands was first normalized to the intensity of the corresponding nonphosphorylated bands, and the ratio was normalized to the control. *Significantly different from Con, $P < 0.05$. †Significantly different from 0.5 day, $P < 0.05$. #Significantly different from 1 day, $P < 0.05$. Solid horizontal bar indicates a significant difference between the soleus and plantaris at $P < 0.05$.

Changes in HSP25 phosphorylation state. For pHSP25 in both the soluble and insoluble fractions, there were significant main effects for days of FO ($P < 0.0001$ and $P < 0.0001$, respectively) and for muscle examined ($P < 0.0001$ and $P < 0.0001$, respectively) and a significant interaction effect for days of FO and muscle examined ($P < 0.0001$ and $P < 0.0001$, respectively). The pHSP25/HSP25 ratio in the soluble fraction was significantly higher than Con at all time points in the plantaris and at 2–7 days of FO in the soleus (Fig. 2*B*). In addition, the response was significantly greater in the plantaris than the soleus at all time points. In the insoluble fraction, the pHSP25/HSP25 ratio was significantly higher than Con in both muscles at 0.5–2 days and similar to Con thereafter (Fig. 3*B*). There was a progressive decrease in the pHSP25/HSP25 ratio

over time in both muscles, such that the values at 1 day of FO were significantly lower than at 0.5 days, and the values thereafter were significantly lower than at both 0.5 and 1 day of FO. In addition, the response was significantly greater in the plantaris than the soleus at 0.5 days.

Changes in TNF-α protein concentrations. There were significant main effects for days of FO ($P < 0.0001$) and for muscle examined ($P < 0.0001$) and a significant interaction effect for days of FO and muscle examined ($P = 0.0098$). At 0 day (Con), the TNF-α levels were similar for the plantaris and soleus (Fig. 4). The TNF-α levels in the plantaris at 0.5–2 days of FO were significantly higher than at 0 day, and thereafter they returned to (3 days) or were lower than (7 days) Con levels. The levels of TNF-α in the soleus were similar to Con from 0.5 to 2 days, and then after 3 or 7 days of FO, TNF-α levels were significantly lower than the levels observed at the earlier time points. In addition, the TNF-α levels were significantly higher in the plantaris than the soleus at 0.5–3 days of FO.

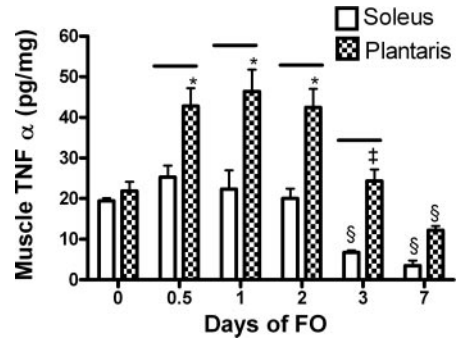

Fig. 4. Time course of the average changes in tumor necrosis factor-α (TNF-α) protein concentration in the plantaris and soleus muscles after 0 (Con), 0.5, 1, 2, 3, and 7 days of FO. *Significantly different from Con, $P < 0.05$. ‡Significantly different from 0.5–2 days, $P < 0.05$. §Significantly different from all other time points, $P < 0.05$. Solid horizontal bar indicates a significant difference between the soleus and plantaris at $P < 0.05$.

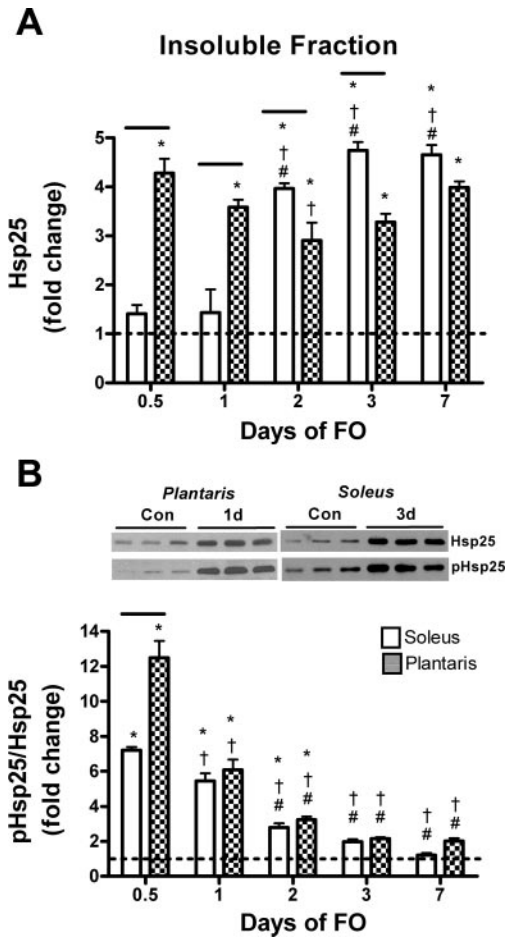

Fig. 3. Time course of the average relative changes (fold change from control) in HSP25 protein (*A*) and pHSP25 (*B*) in the insoluble fraction in the plantaris and soleus muscles after 0.5, 1, 2, 3, and 7 days of FO. In both graphs, the control value is set at 1.0 as indicated by the dashed line. Representative Western blots with 3-Con and 3-1d FO plantaris or 3-Con and 3-3d FO soleus muscle samples are shown. The intensity of the phosphorylated bands was first normalized to the intensity of the corresponding nonphosphorylated bands, and the ratio was normalized to the control. *Significantly different from Con, $P < 0.05$. †Significantly different from 0.5 day, $P < 0.05$. #Significantly different from 1 day, $P < 0.05$. Solid horizontal bar indicates a significant difference between the soleus and plantaris at $P < 0.05$.

TNF-α stimulates HSP25 phosphorylation in C_2C_{12} myotubes. Exposure of C_2C_{12} cells to TNF-α resulted in a time-dependent increase in HSP25 phosphorylation via activation of the MAP kinase pathway (Fig. 5). TNF-α increased HSP25 phosphorylation at 15, 30, and 60 min, with maximal phos-

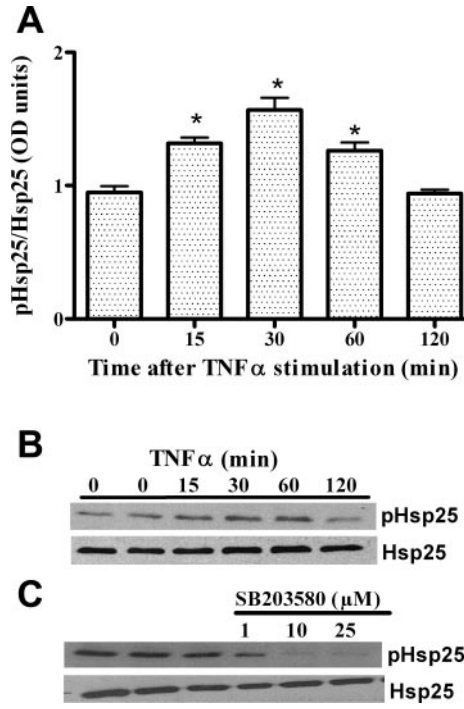

Fig. 5. *A*: time course of average changes in pHSP25 in C_2C_{12} myotubes following the addition of 10 ng/ml recombinant murine TNF-α. The intensity of the phosphorylated bands was first normalized to the intensity of the corresponding nonphosphorylated bands. *B*: representative Western blots demonstrating that TNF-α increased phosphorylation of HSP25 at early time points before returning to basal levels after 120 min. *C*: representative Western blots showing that treatment with SB-203580 (10 or 25 μM) blocks phosphorylation of HSP25 after 30 min of TNF-α stimulation without affecting HSP25 expression. In all lanes, cells were treated with 10 ng/ml of TNF-α, while *lanes 3–6* were pretreated with 1, 10, or 25 μM of SB-203580, 30 min before the addition of TNF-α. Values are means ± SE; $n = 3$ experiments per time point. OD, optical density. *Significantly different from control (*time 0*), $P < 0.05$.

phorylation observed at 30 min (Fig. 5*A*). After 120 min, pHSP25 had returned to control levels. HSP25 protein levels were unchanged in response to TNF-α. Phosphorylation of HSP25 by TNF-α was blocked in myotubes by treatment with the p38 MAP kinase inhibitor, SB-203580 (10 μM), without affecting HSP25 expression (Fig. 5*B*).

DISCUSSION

The present results provide the first evidence that HSP25 is rapidly phosphorylated and shifts to the insoluble fraction during the early stages of muscle adaptation to abrupt increases in loading and activation, particularly in the fast plantaris. This significant increase in pHSP25 in the insoluble fraction suggests early stabilization of cytoskeletal elements (22) because this function likely requires large amounts of phosphorylated HSP25 (32). The higher levels of TNF-α in the plantaris relative to the soleus suggest that TNF-α contributes to the greater pHSP25 response in the soluble fraction in the plantaris as TNF-α increased HSP25 phosphorylation in isolated muscle cells.

Changes in HSP25 mRNA levels. Following FO, the early increases in HSP25 mRNA indicate that increases in gene transcription likely contributed to the later elevations in HSP25 protein observed in both the plantaris and soleus. The increases in HSP25 mRNA observed after FO are more rapid and of a greater magnitude than previously reported in a model of increased muscle activation, i.e., chronic electrical stimulation (34). In the chronically stimulated (6–10 Hz, 24 h per day) rabbit tibialis anterior, HSP25 mRNA was unaffected after 8 h, 1 day, or 3 days; began to increase after 7 days; and was significantly increased above control levels after 14 and 21 days. In contrast, in the present study, the greatest induction of HSP25 mRNA was observed at the early time points and then was attenuated with continued FO in both the soleus and plantaris. Different responses in these two models, however, were not unexpected, because FO results in an increase in both activation and loading, whereas low-frequency chronic stimulation increases muscle activation only. FO subjects a muscle to high loads and results in eventual muscle hypertrophy (2, 3, 25, 45), whereas chronic stimulation increases muscle activation with little or no additional loading and often results in muscle atrophy (8, 41). Furthermore, FO is likely associated with greater muscle damage (5, 50) than low-frequency stimulation. The important role of muscle damage and loading in increasing HSP25 mRNA is further evidenced by the observation that a single bout of high-force eccentric contractions resulted in a 135% increase in HSP25 mRNA 48 h postexercise in the human biceps brachii (51).

Changes in HSP25 protein and phosphorylation state. These results demonstrate that the HSP25 response to muscle overload is initiated within 0.5 days, particularly in the insoluble fraction that showed increases in pHSP25 in both muscles and HSP25 in the plantaris. Rapid changes in the soluble fraction included a sixfold increase in pHSP25 in the plantaris. It was important to study HSP25 in both fractions because previous work has demonstrated translocation of HSP25 to the insoluble fraction in response to various stressors, including muscle damage (22) and the accumulation of damaged proteins in cardiomyocytes (55). Changes in total HSP25 protein in the soluble fraction are not evident until 2 days after FO. In humans, 2 days of chronic muscle overload may not be necessary for increases in HSP25 because a single bout of high-intensity resistance training was associated with increased HSP25 48 h postexercise (51, 52). Increased levels of HSP25 in overloaded muscles are maintained for at least 7 days in both the rat soleus and plantaris muscles (15) and during stretch hypertrophy in the quail anterior latissimus dorsi muscle (49).

Although HSP25 increased in both muscles, the response in both the soluble and insoluble fractions was muscle specific. In the soluble fraction, the increase in HSP25 was significantly greater in the plantaris than the soleus at the later time points, i.e., after 3 and 7 days of FO. Our laboratory (15) and others (13, 31) have suggested that the HSP response to a muscle stressor (i.e., overload, exercise) may be related to the basal level of HSP expression in the muscle. In the present study, the greater response in the fast plantaris compared with the slow soleus may be due in part to the higher basal expression of HSP25 in muscles composed of predominately slow MHC compared with muscles with predominately fast MHC profiles (17, 19, 34). Specifically, our laboratory previously reported that HSP25 protein levels were ~2.5 fold higher in the slow soleus and adductor longus than the fast plantaris (17). At the mRNA levels, HSP25 mRNA was approximately threefold higher in the soleus than the fast (white) region of the vastus (34). The greater responsiveness in muscles with lower unstressed HSP25 content (i.e., plantaris) is supported by a recent study in humans suggesting that the basal level of HSP expression (HSP72 and HSP27) in skeletal muscle may contribute to the "regulation" of HSP expression with exercise training (13). Furthermore, Morton et al. (31) suggested that the absence of an increase in HSP27 following nondamaging treadmill running in humans may been related to its high baseline expression in the vastus lateralis.

The relative increase in HSP25 in the insoluble fraction was greater than the response in the soluble fraction at early time points in the plantaris and at the later time points in the soleus (cf. Figs. 2*A* and 3*A*). The significantly greater increase in HSP25 in the insoluble fraction in the plantaris compared with the soleus does not appear to be due to increased transcription as the mRNA responses are similar between muscles. Thus further research is needed to determine whether factors such as increased mRNA stability, translation efficiency, and/or reduced protein degradation may have contributed to these findings. However, these early increases in HSP25 in the insoluble fraction in a fast muscle are consistent with previous work demonstrating a dramatic shift of HSP25 from the soluble to the insoluble fraction immediately after a bout of lengthening contractions in the rat extensor digitorum longus, a predominantly fast digit extensor (22). Immediately after the muscle damage induced by lengthening contractions, HSP25 translocated from a diffuse cytosolic location to striations corresponding to the Z disks (22). This observation suggests that the greater early increase in HSP25 and pHSP25 in the insoluble fraction in the plantaris relative to the soleus may be related to more immediate muscle damage in the plantaris and, therefore, an increased need to limit cytoskeletal damage, remove damaged proteins, and assist in repair. This possibility is consistent with the observation that fast fibers are more susceptible to eccentric-induced muscle damage than slow fibers (11, 27). Interestingly, following nondamaging treadmill running, the protein content of both HSP70 and HSP60 increased in the

human vastus lateralis, whereas HSP27 was unchanged, leading the investigators to suggest that HSP27 is more sensitive to structural or functional damage than the larger HSPs because their protocol did not induce muscle damage (31).

Importantly, the translocation of HSP25 to the insoluble fraction following damaging contractions was associated with increased phosphorylation (22). The increased phosphorylation of HSP25 may be important for stabilizing cytoskeletal elements as shown in other cell types, such as fibroblasts (14, 18, 23, 24). It has been proposed that in unstressed cells, nonphosphorylated HSP25 forms large aggregates; however, one of the earliest responses to stress is HSP25 phosphorylation, which disrupts the large aggregates allowing HSP25 to interact directly or indirectly with F-actin and protect it from damage and actin-severing proteins as well as facilitate subsequent reorganization (32, 57). In muscle cells, the functional significance of HSP25 phosphorylation is not well understood, and limited data are available. However, based on our present work and other work in skeletal (22) and cardiac (7) muscle, increased HSP25 phosphorylation appears to play a role in the muscle remodeling processes associated with muscle damage. We observed substantial early increases in total pHSP25 in the insoluble fraction in both muscles, and from a functional standpoint this may contribute to maintaining muscle integrity as the muscle adapts to the sudden increases in muscle loading and activation. The subsequent decline in pHSP25 in the insoluble fraction with continued overload suggests that ongoing muscle remodeling may reduce the need for phosphorylation-dependent cytoskeletal stabilization.

HSP25 also may protect against the cytotoxic effects associated with the proinflammatory cytokine, TNF-α. During the initial stages of FO, an inflammatory response is initiated, and it is still unclear if this response is physiologically beneficial (54). Whereas some inflammation ultimately promotes muscle regeneration and remodeling, excess inflammation is likely detrimental to muscle remodeling. Macrophages can infiltrate overloaded skeletal muscles and release proinflammatory cytokines, such as TNF-α and interleukin-1β (53). Skeletal muscle TNF-α mRNA is increased fourfold in the gastrocnemius of young rats after 3 days of overload (53). We have shown significantly higher TNF-α levels in the plantaris relative to the soleus at all time points except 7 days, consistent with previous reports of higher TNF-α expression in fast type II than slow type I fibers in both humans (43) and rats (42). However, evidence suggests that the cytotoxic effects of TNF-α may be reduced by elevated levels of HSP25. Specifically, overexpression of HSP25 was associated with significant reductions in TNF-α-induced oxidative damage in L929 fibroblasts (40, 44). In addition, protection against the cytotoxic effects of TNF-α were reduced in a dose-dependent fashion by increasing HSP25 expression in L929 cells (30). Interestingly, L929 cells, which are highly sensitive to TNF-α-induced damage, do not seem to express HSP25 (26).

Although the protective actions of HSP25 against TNF-α-induced damage may not involve increased phosphorylation (44), TNF-α is associated with increased pHSP25 in muscle cells (present in vitro results) and other cell types (9, 39). This increase in pHSP25, however, may serve to stabilize cytoskeletal elements and maintain muscle integrity because these functions appear to be phosphorylation dependent (32, 57). The close temporal relationship between elevated TNF-α and pHSP25 in the plantaris relative to the soleus suggests that TNF-α contributes to the significantly greater pHSP25 response in the plantaris (soluble fraction).

HSP25 is one member of a large family of HSPs that may contribute to the adaptation of skeletal muscle to overload stress. We recognize that the physiological functions of HSP25 may be impacted by other HSPs, in particular, the other small HSPs, αβ-crystallin and HSP20 (57). Furthermore, several studies to date have shown significant increases in HSP70 (HSP72) after 14–28 days in the overloaded rat plantaris (36–38) and increases in HSP70 within 48 h of a bout of eccentric exercise in humans (51, 52).

In summary and conclusion, we have presented novel results demonstrating the earliest reported changes in HSP25 localization and phosphorylation and in the proinflammatory cytokine, TNF-α, associated with increases in muscle loading and activation. Importantly, the differences in the responses between the predominantly slow soleus and the predominantly fast plantaris highlight the variability in the stress experienced by each muscle during adaptation to FO. One such difference was the elevation in TNF-α, which may be a mechanism contributing to the greater pHSP25 response in the soluble fraction in the plantaris compared with the soleus after 0.5, 1, 2, and 3 days of FO. Strong support for this possibility is the close temporal association with the highest levels of TNF-α and pHSP25 in the plantaris and the phosphorylation of HSP25 by TNF-α in isolated skeletal muscle cells.

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