Attenuation of unloading-induced rat soleus atrophy
with the heat-shock protein inducer 17-(allylamino)-
17-demethoxygeldanamycin

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ABSTRACT We hypothesized that pharmacological induction of HSP70 would attenuate soleus atrophy development under 3 d of rat hindlimb unloading. Male Wistar rats were divided into control (C; n=7), 3-d hindlimb unloading (HUL; n=7), HUL with HSP90 inducer administration, 17-allylamino-17-demethoxygel-danamycin (17-AAG; 60 mg/kg, HUL+17-AAG, n=8). The relative weight of soleus muscle to body weight [soleus wt (mg)/body wt (g)] in the HUL group was less than that of the C and HUL + 17-AAG groups (P<0.05). We revealed HSP90, HSP70 mRNA decrease in the HUL group (but not the HUL + 17-AAG group) vs. C (P<0.05). The unloading resulted in significant increases of µ-calpain and conjugated ubiquitin (Ub) levels (proteins as well as mRNAs) vs. the C group, whereas 17-AAG administration prevented these alterations (studied by SDS-PAGE and RT-PCR). pFOXO3 protein was decreased in the HUL group vs. C, but not in HUL + 17-AAG. Content of E3-lygase (MuRF-1, MAFbx) mRNA was increased in both suspended groups. In summary, 17-AAG administration attenuates soleus muscle atrophy, µ-calpain, and Ub increases under hindlimb unloading as well as decrease of pFOXO3.—Lomonosova, Y. N., Shenkman, B. S., Nemirovskaya, T. L. Attenuation of unloading-induced rat soleus atrophy with the heat-shock protein inducer 17-(allylamino)-17-demethoxygeldanamycin. FASEB J. 26, 4295–4301 (2012). www.fasebj.org

HEAT-SHOCK PROTEINS (HSPs) ARE SYNTHESIZED IN GREAT AMOUNTS IN SKELETAL MUSCLE; THEIR LEVELS INCREASE RAPIDLY FOLLOWING A VARIETY OF STRESSES TO PROVIDE CYTOPROTECTION (1). HOWEVER, HSP90 AND HSP70 LEVELS DRAMATICALLY DROP BY 70–75% UNDER MUSCLE UNLOADING IN PARALLEL WITH MUSCLE ATROPHY (2, 3). PROBABLY, THAT INABILITY TO PRODUCE HSPs HAS AN IMPORTANT EFFECT ON MUSCLE ATROPHY DURING UNLOADING. ALTERING OF CHAPERONE FUNCTIONS IS ASSOCIATED WITH DEVELOPMENT OF SEVERAL DISEASES. RECENTLY, IT WAS SHOWN THAT PLASMID-MEDIATED OVEREXPRESSION OF HSP70 IN THE SOLEUS MUSCLE OF RATS WAS SUFFICIENT TO ATTENUATE SKELETAL MUSCLE ATROPHY IN THE FIBERS (3). HOWEVER, THE MECHANISM THAT REGULATES THE SYNTHESIS OF HSPs IN UNLOADED MUSCLE IS UNKNOWN. WE ASSUMED THAT UNDER MUSCLE UNLOADING, HSP90 FUNCTION, WHICH LEADS TO HSP70 SYNTHESIS PRECLUSION, MIGHT BE CHANGED. WE HAVE TESTED THE HYPOTHESIS THAT THE HSP90 inducer 17-(allylamino)-17-demethoxygel-danamycin (17-AAG) CAN PREVENT HSP70 DECREASE AND ATTENUATE ATROPHY OF UNLOADED SOLEUS DUE TO MODULATION OF HSP90 FUNCTION. 17-AAG, WHICH SPECIFICALLY BINDS TO THE HSP90 ATP-binding site and disrupts its ATP-dependent function, is known to activate HSP90 nonspecifically by the activation of heat-shock factor 1 (HSF1; refs. 4, 5), thereby causing the activation of the heat-shock or stress-response pathways. In the resting cells, several chaperones, particularly HSP90, were shown to bind to HSF1 and keep it in an inactive form. During stress (and exercise activity), these repressing chaperones become occupied by misfolded proteins, which resulted in the dissociation of the cytoplasmic chaperone/HSF1 complex (4, 5). During unloading, we administered HSP90 inducer 17-AAG to disrupt ATP-dependent function and to increase the HSP amounts. The dissociation of HSF1 from HSP90 allows its translocation to the cell nucleus. Part of the nuclear HSF1 is assembled to heat shock granules, which may modify the chromatin structure (6). Binding of HSF1 to the heat-shock elements of the heat-shock-inducible genes unlocks the RNA polymerase, being arrested (paused) in most of these genes after transcribing the initial segment of the mRNA in the absence of HSF1. On the other hand, as was demonstrated earlier, the molecular chaperone HSP90 may regulate Akt-dependent signal pathways, and formation of the Akt-HSP90

Abbreviations: 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; C, control; FOXO3, Forkhead box O 3; HSF1, heat-shock factor 1; HSP, heat-shock protein; HUL, hindlimb unloading; MAFbx, muscle atrophy F-box; MuRF-1, muscle-specific RING finger 1; p70S6k, p70S6 kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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complex stabilized the Akt kinase activity (7). These researchers demonstrated that Akt binding to HSP90β was essential for Akt phosphorylation, since reduced HSP90 levels upon muscle unloading might cause Akt decline or inactivation. Akt activates downstream targets to mediate protein synthesis, but muscle disuse reduces Akt activation below control levels (8). Because an important element of Akt anabolic and catabolic signaling may be associated with HSP90 levels, we tested phosphorylated Akt and p70S6 kinase (p70S6k) levels in soleus of hindlimb-suspended and 17-AAG-treated rats, as well as one from a downstream target of the PI3K/AKT pathway, the Forkhead box O 3 (FOXO3) transcription factor. Its dephosphorylation by Akt leads to nuclear entry and growth suppression. Thus, catabolic signaling is the subject of regulation by HSPs as well (9). However the mechanism for this process during muscle unloading has not been studied sufficiently. We have investigated the key markers of the initial and final links of the catabolic process: calcium-activated protease μ-calpain and E3 ligases, muscle atrophy F-box (MAFbx), muscle-specific RING finger 1 (MuRF-1), and conjugated ubiquitin, which represented the ubiquitin-proteasome pathway. Thus, additional goals of the present experiments were to determine the effect of 17-AAG treatment on key chains of the anabolic and catabolic signaling pathways. If our hypothesis is valid, blocking of the ATP-binding site and disruption of its ATP-dependent function should nonspecifically induce HSP syntheses and attenuate muscle atrophy.

MATERIALS AND METHODS

Animal procedures

The experiment was carried out in accordance with the rules of biomedical ethics (protocol 264, March 5, 2011) certified by the Russian Academy of Sciences Committee on Bioethics. All animals were kept at 22°C; water and food for rodents were available ad libitum. Twenty-two young adult male Wistar rats weighing 191.1 ± 3.2 g were randomly assigned to the ground control (C) group (n = 7), the hindlimb unloading (HUL) group (n = 7), or the HUL with HSP90 inhibitor 17-AAG (60 mg/kg i.p.) administration (HUL + 17-AAG) group (n = 8). Geldanamycin (GA; cat. no. A-6880; LC Laboratories, Woburn, MA, USA) was dissolved in 20% dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA) in 0.9% saline. The tissue distribution, pharmacokinetics, and doses of 17-AAG in rats and mice were described previously (10, 11, 12). Control animals received identical volumes of 20% DMSO vehicle. 17-AAG and vehicle were administered 3 h prior to the start of the experiment. Control animals were housed in groups of 3 in a temperature- and light-controlled environment (i.e., 12-h light-dark cycle). HUL was carried out for 3 d. At the end of the experiment, rats were euthanized, and the muscles were rapidly removed, weighed, and frozen at −85°C for later analysis. Control animals were processed along with the HUL and HUL + 17-AAG animals.

Hindlimb-suspension protocol

The HUL model was used to employ a tail-traction method of noninvasive tail-casting procedure, as described previously (13). The technique used a swivel harness system incorporated into the casting materials, which was attached to a hook at the top of the cage. The hook was adjusted to allow only the forelimbs of the animal to reach the floor of the cage with the hindlimbs suspended; the body axis was at a 45° angle to the cage floor. Suspended animals were free to move about the cage using their forelimbs to obtain food and water.

Protein extraction and Western blot analysis

Skeletal muscle tissue (30 mg) was homogenized in ice-cold RIPA lysis buffer, as described previously (14): 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, and 5 mM EDTA, supplemented with 1 mM DTT, 1 mM PMSF, and 1 mM pepstatin (Sigma-Aldrich, St, Louis, MO, USA), mammalian protease inhibitor cocktail (Amresco, Solon, OH, USA), and phosphatase inhibitor cocktail B (Santa Cruz Biotechnol, Santa Cruz, CA, USA). The total protein concentration of the lysates was determined by incubation for 20 min at 4°C and centrifugation for 10 min at 12,000 rpm. Protein content of supernatants was quantified using an assay based on a modification of the Lowry protocol (RC DC Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin was used as a standard. The samples were diluted in Laemmli buffer. Total protein (20-50 μg) was subjected to SDS-PAGE, and the proteins were then transferred to nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked for 1 h at room temperature with blocking buffer (4% nonfat milk powder; TBS, pH 7.4; and 0.1% Tween 20) and incubated overnight at 4°C with primary monoclonal antibodies against calpain 1 (diluted 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), ubiquitin (diluted 1:500; Abcam, Cambridge, MA, USA), FOXO3 diluted 1:700; Santa Cruz, Biotechnology), pFOXO3 phosphorylated at Ser-253 (diluted 1:700; Santa Cruz Biotechnology), Akt phosphorylated at Ser-473 (diluted 1:400; Cell Signaling, Danvers, MA, USA), p70S6k phosphorylated at Thr-389 (diluted 1:2000; Santa Cruz Biotechnology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; diluted 1:5000; Abcam, Cambridge, MA, USA). Antibodies were diluted in the blocking buffer. Three 10-min washes with TBS-Tween (TBS and 0.1% Tween 20) were then performed, after which membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse immunoglobulins (diluted 1:2000; Bio-Rad Laboratories). The membranes were washed again in TBS-Tween 3 times for 10 min, incubated in Immun-Star HRP Chemiluminescent system (Bio-Rad Laboratories), and exposed to X-ray film (Eastman Kodak, Rochester, NY, USA); the images were then scanned. The protein bands were quantified using densitometry scanning (GS-800; Quantity-One software, Bio-Rad Laboratories). Protein density was normalized to GAPDH, and the relative density of the control group band was taken as 100%. Changes were calculated as percentage of control level for each group.

RNA analysis

Total RNA was extracted from 10 mg frozen muscle soleus using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA samples were treated by proteinase K and DNase I. RNA concentration was determined at 260 nm. For storage, aqueous solution of isolated RNA was frozen at −84°C. For reverse
transcription to be provided, 1 µg RNA, oligo(dT)$_{15}$, random hexamers d(N)$_{6}$, dNTPs, RNase inhibitor, and reverse transcriptase MMLV (60 min at 37°C) were used. For each target mRNA, 1 µl cDNA was amplified in a 25 µl SYBR Green PCR reaction containing 1× Quantitect SYBR Green Master Mix (Syntol) and 10 µM of each primer: 5'-GAGCAAGGAGAAAGTG-3' and 5'-ATGGCATTCTGCAGTTC-3' for HSP90β; 5'-GCACGATTCTGCAGTTC-3' and 5'-GGCGGAGAACATTCC-3' for HSP70; 5'-GAGCAAGGAGAAAGTG-3' and 5'-ATGGCATTCTGCAGTTC-3' for GAPDH; 5'-GGCAATTGTTGCTTTTGT-3' and 5'-AAATTCAGTCC-3' for calpain 1; 5'-GGCGGAGAACATTCC-3' and 5'-CACTGCTGCTGAGGAGG-3' for MAFbx; 5'-GAGCAAGGAGAAAGTG-3' and 5'-ATGGCATTCTGCAGTTC-3' for pFOXO3 and FOXO3 in whole-tissue lysate. Decrease of pFOXO3 was found in the HUL group compared with the C group. At the same time, 17-AAG treatment (HUL+17-AAG group) led to a much greater level of mRNA HSP90 and HSP70 expression relative to the HUL group.

Muscle weight

The C group was analyzed in parallel with the HUL and HUL + 17-AAG experimental groups. Because the body and muscle weights of both suspended groups after cessation of the study tended to be different from the control values (ranging from 4 to 6%), we focused on the relative muscle mass, which is the ratio of muscle weight to body weight, to correct for this change (Fig. 2). In any case, 17-AAG did not cause a reduction in body weight in the treated group. The relative soleus muscle mass in the HUL group was significantly lower than that in the control (Fig. 2). However, in parallel with the increase of HSP mRNA, soleus atrophy was attenuated after 3 d of suspension in the 17-AAG-treatment group, since there was no difference in the relative weight of the soleus muscle between this group and control animals.

Catabolic markers

To test whether atrophy prevention in the HUL + 17-AAG group was associated with alterations in FOXO3 phosphorylation, we measured the amount of pFOXO3 and FOXO3 in whole-tissue lysate. Decrease of pFOXO3 was found in the HUL group vs. C group (46%), but not in the HUL + 17-AAG group (Fig. 3). At the same time, the total levels of FOXO3 were equal in all experimental groups (Fig. 3). But we were surprised to see the great increase in MAFbx and MuRF-1 (E3 ligase) mRNA expression in both suspended groups in comparison with the control group (Fig. 4).

Statistical analysis

All data were expressed as means ± se. Significant differences between groups were statistically analyzed using 2-way ANOVA followed by Tukey’s test. When normality testing failed, data were analyzed by nonparametric methods (Kruskal-Wallis ANOVA followed by Dunnett’s test). Differences with values of $P < 0.05$ were considered to be statistically significant.

RESULTS

mRNA HSP90β and HSP70 content

Fig. 1A, B shows a profound decrease of both HSP90β and HSP70 mRNA expression under suspension in the HUL group compared with the C group. At the same time, 17-AAG treatment (HUL+17-AAG group) led to a much greater level of mRNA HSP90 and HSP70 expression relative to the HUL group.

Figure 1. Changes in HSP90 (A) and HSP70 (B) mRNA contents in musculus soleus of HUL (n=7) and HUL+17-AAG (n=8) groups were estimated relative to C group (n=7) by the Livak technique. Data are expressed as means ± se. *$P < 0.05$ vs. C; **$P < 0.05$ vs. HUL + 17-AAG.

Figure 2. Ratio of muscle weight to body weight in C (n=7), HUL (n=7), and HUL+17-AAG (n=8) groups. Data are expressed as means ± se. *$P < 0.05$ vs. C; **$P < 0.05$ vs. HUL + 17-AAG.
But despite similar amounts of expression of E3 ligases MAFbx and MuRF-1 mRNA in both hindlimb-suspension groups, the levels of ubiquitin-conjugated proteins determined in soleus cytosolic fractions (Fig. 5) of the HUL + 17-AAG group was significantly diminished in comparison with the HUL group. Nevertheless, the levels of ubiquitin-conjugated proteins in both hindlimb-suspension groups exceeded the control level. Sufficient increase of μ-calpain at both protein and mRNA levels was revealed only in HUL group (in parallel with the soleus atrophy and the level of ubiquitin-conjugated proteins rising) as compared with the C and HUL + 17-AAG groups (Fig. 6).

**DISCUSSION**

The main goal of this study was to investigate the involvement of HSP90 in anabolic and catabolic signaling alterations under conditions of muscle unloading. In previous findings, it was suggested that muscle atrophy on unloading could be caused by a decrease in HSP levels (3, 16, 17, 18). However, it remains unknown which signaling pathways are controlled by HSP90 under muscle unloading. To answer this question, some researchers have increased HSP levels by means of plasmid-mediated HSP70 overexpression (3) to prevent the fiber atrophy. Other researchers used HSF1-null mice to analyze the effect of the HSFI gene on HSP production under conditions of muscle atro-
phy and regrowth (18). We are not aware of any previous findings that have directly addressed HSP90 under muscle unloading in vivo to investigate its function and signaling control. The HSP we are interested in, HSP90, has emerged as a key factor in the regulation of HSF1. It is known that 17-AAG treatment induces up-regulation of HSP70 and other proteins through the action of the transcription factor HSF1 (19). These newly synthesized proteins may work to restore cellular homeostasis under muscle unloading. Significantly lower expression of HSP90/HSP70 mRNA in the soleus muscle was observed after 3 d of HUL in this study. This phenomenon may be accounted for by the decrease in the relative weight of the soleus muscle. The recent finding suggests that the relative muscle mass reduction occurs during the early (1 and 5 d) stages of unloading (20). At the same time, 17-AAG administration (HUL/H11001 17-AAG group) led to much greater amounts of not only HSP90 but also HSP70 mRNA compared with the hindlimb-suspension group. The increase in HSP70 expression has been shown previously in response to 17-AAG administration (19). In parallel to the increase of HSP90/HSP70 mRNA expression, soleus atrophy was prevented. The muscle atrophy caused by unloading is the result of both reduced protein synthesis and increased protein degradation, but it appears that the latter plays the larger role (21). Protein degradation is mediated primarily through the ubiquitin proteasome pathway (22, 23). There are two commonly used markers of this pathway: the muscle-specific E3 ligases, atrogin-1/MAFbx and MuRF1. Expression of these ligases increased severalfold during early stages of muscle atrophy, and they catalyzed the transfer of active ubiquitin to specific protein substrates (23). We were surprised at the great increase (Fig. 4) of MAFbx and MuRF-1 mRNA expression in both suspension groups, the levels of ubiquitin-conjugated proteins in the HUL + 17-AAG group were significantly diminished in comparison with the HUL group (Fig. 5). There could be several explanations of this result. The regulation of ubiquitiniza-

Figure 6. Changes in μ-calpain mRNA (A) and μ-calpain protein (B, C) contents in musculus soleus of HUL (n=7) and HUL+17-AAG (n=8) groups were estimated relative to C group (n=7). Data are expressed as means ± se. *P < 0.05 vs. C; #P < 0.05 vs. HUL + 17-AAG.

Figure 7. A) Protein expression of phospho-Akt (Ser-473). B) Data are expressed as mean ± se percentage of C group (n=7) from HUL (n=7) and HUL+17-AAG (n=8) groups. *P < 0.05 vs. C.

Figure 8. A) Protein expression of phospho-p70S6k (Thr-389). B) Data are expressed as mean ± se percentage of C group (n=7) from HUL (n=7) and HUL+17-AAG (n=8) groups.
tion may be realized not only by means of MAFbx and MuRF-1 E3 ligases, but via other E3 ligases. It was shown recently that HSP70 can inhibit NF-κB activation in skeletal muscle, either in vitro or in vivo (3). It is worth noting that the HSP70 mRNA level in the HUL + 17-AAG group was significantly higher than that in the HUL group. Furthermore, recent observation has demonstrated that HSP90 interacts very dynamically with a variety of proteins that are not classic HSP90 clients, and this dynamic cycling of HSP90 protects from CHIP-mediated ubiquitination (24). We may suppose that the progressive events in unloaded muscle result in the opening of the ligand-binding cleft of the client protein as the initial step in protein unfolding, and as long as HSP90 can interact to stabilize the cleft, it will buffer the effect of unloading damage. When the cleft is opening, such that HSP90 can no longer interact, HSP70-dependent ubiquitination occurs. As HSP90 mRNA levels in the HUL + 17-AAG group exceed those not only of the HUL but also the C group, this mechanism of protein protection can take place. In addition, there is a SUMO pathway that may regulate deubiquitination of proteins (25) and be involved in preventing ubiquitin-conjugate accumulation. Significant elevation of μ-calpain at both protein and mRNA levels was revealed only in the HUL group (in parallel with the soleus atrophy and the rising levels of ubiquitin-conjugated proteins) as compared with the C and HUL + 17-AAG groups. The activity of ubiquitous calpains (μ-and m-calpain) and muscle-specific (calpain-3) proteases depends on the calcium concentration (Fig. 6). It was demonstrated earlier that the increase in calcium concentration under HUL of rats could trigger the increase of μ-calpain levels (26). However, the mechanism that may lead to the prevention of μ-calpain increase in the HUL + 17-AAG group is unknown. It has been proposed that calpain activation may inhibit the anabolic signaling of Akt, since a molecular chaperone previously shown to mediate Akt activity, HSP90, is a calpain substrate (15). However, explanation of the opposite effect, how the activation of HSP90 can lead to reduced μ-calpain expression, has not been demonstrated.

The expression of atrogin-1/MAFbx may be regulated by the transcription factor FOXO, which is normally phosphorylated and is thus inactivated by Akt/ PKB, but translocates to the nucleus and induces the transcription of both atrogin-1 and MuRF1 in the absence of Akt repression (27, 28, 29). In parallel with E3-ligase examination, we determined pFOXO3 and FOXO3 values. Contrary to elevated E3-ligase mRNA expression levels in both hindlimb-suspension groups, pFOXO3 level in the HUL group was reduced in comparison with control (Fig. 3). At the same time, the 17-AAG-treated group (HUL + 17-AAG) avoided this decline. FOXO3 protein level remained the same in all groups. It is evident that pFOXO3/FOXO3 in our case could not regulate MAFbx and MuRF1 expression in the HUL + 17-AAG group. There is evidence of one more way to control the expression of these E3 ligases, by means of myogenin. Myogenin is up-regulated in skeletal muscle following denervation (30), and beneficial effects of 17-AAG on spinal muscle atrophy were also found (12). We found equal amounts of pAkt in control and HUL + 17-AAG groups (in parallel to pFOXO3 levels; Fig. 7), but pAkt levels in the HUL group were diminished. Modulation of HSP90 function by 17-AAG treatment lends support to the suggestion that anabolic signaling and phosphorylation of the FOXO3 transcription factor are controlled by Akt. Soleus atrophy attenuation in the HUL + 17-AAG group was not associated with phosphorylation of p70S6k, because its level was equal in all three groups of rats (Fig. 8). Akt-mTOR-S6K is well known as the pathway of protein synthesis regulation at the level of translation initiation (8). However, it should be noted that in addition to Akt-mTOR-S6K, there are different factors involved in the regulation of protein synthesis, like those at the level of translation or elongation.

In summary, we showed that 17-AAG administration (enhancing HSP90 expression) under HUL conditions attenuated soleus atrophy and prevented HSPs mRNA drop, maintained p-Akt and p-FOXO3 content at the control level. Moreover, it was found that prevention of ubiquitination and μ-calpain expression were increased in the 17-AAG-treatment group.

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