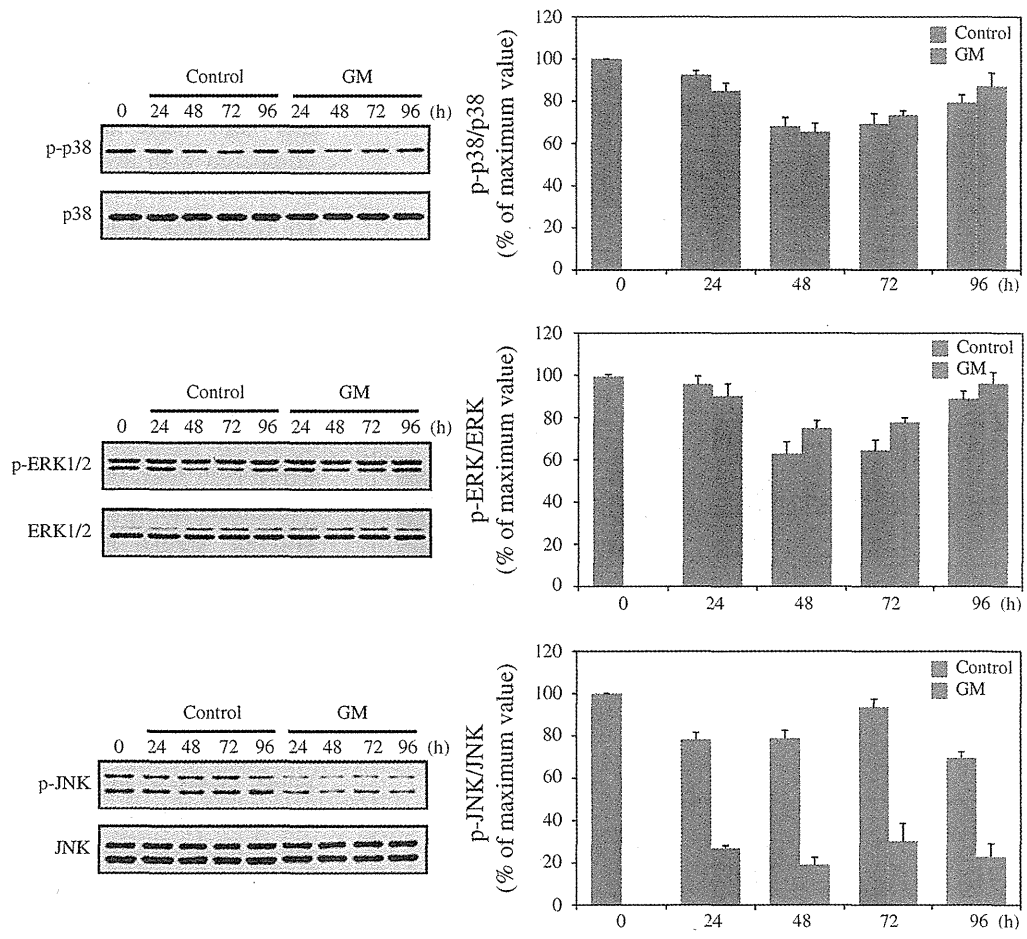


Fig. 4 Effects of geldanamycin treatment on levels of phosphorylated p38MAPK$^{Thr180/Tyr182}$, ERK1/2$^{Thr202/Tyr204}$, and JNK$^{Thr183/Tyr185}$ proteins during myogenic differentiation. Histograms represent the temporal changes in levels of p38MAPK$^{Thr180/Tyr182}$/p38MAPK, phosphorylated ERK1/2$^{Thr202/Tyr204}$/ERK1/2, and JNK$^{Thr183/Tyr185}$/JNK during myogenic differentiation. The intensities of bands were measured and normalized to the maximum value observed during 4 days in culture. The data are the means ± SD (n = 3/time point)

kinase activity correlated with Ser473 but not Thr308 phosphorylation [31]. Therefore, we examined the effect of geldanamycin on the phosphorylation levels of Akt1^{Ser473} protein during myogenic differentiation (Fig. 5). The phosphorylation levels of Akt1^{Ser473} protein increased to a peak at 72 h, followed by a decrease at 96 h in the untreated C2C12 cells. The treatment of geldanamycin gradually reduced phosphorylation levels of Akt1^{Ser473} protein during myogenic differentiation. The expression levels of Akt1 protein also decreased in the treated C2C12 cells. The ratio of phosphorylated Akt1^{Ser473} to Akt1 was gradually increased in the geldanamycin-treated C2C12 cells. This indicated that the amount of Akt1 protein decreased greater than that of phosphorylated Akt1^{Ser473}, resulting in increasing ratio of pAkt1-^{Ser473} to Akt1.

Pharmacological inhibition of HSP90 activity induces apoptosis during myogenic differentiation

Akt also enhances the survival of cells by blocking the function of proapoptotic proteins and processes [32]. Therefore, decreased phosphorylation levels of Akt1 might be expected to induce apoptotic cell death in C2C12 cells. We examined the frequency of apoptotic nuclei in the C2C12 cells treated with geldanamycin for 96 h using TUNEL method, which marks cells in a relative late phase of apoptosis. TUNEL-positive nuclei were significantly increased in the treated C2C12 cells compared with the untreated C2C12 cells (Fig. 6a). To evaluate whether a deregulated balance between pro-apoptotic and anti-apoptotic proteins could contribute to the observed apoptosis in the treated C2C12 cells, the expression levels of Bcl-2-family proteins were examined. As shown in Fig. 6b, the expression levels of anti-apoptotic Bcl-2 protein in the treated C2C12 cells were very low compared with that in the untreated C2C12 cells. In contrast, the expression levels of pro-apoptotic Bax protein in the treated C2C12 cells were nearly equivalent to that in the untreated C2C12 cells. The ratio of Bcl-2 to Bax is important in determining susceptibility to apoptosis [33]. As expected, the ratio of Bcl-2 to Bax in the treated C2C12 cells was extremely low compared with that in the untreated C2C12 cells.

The geldanamycin derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG), also decreases the expression levels of HIF-1α, myogenic regulatory factors MyoD and myogenin, and Akt1

17-AAG is a less-toxic derivative of the geldanamycin and is now in clinical trial. We used 17-AAG to further confirm

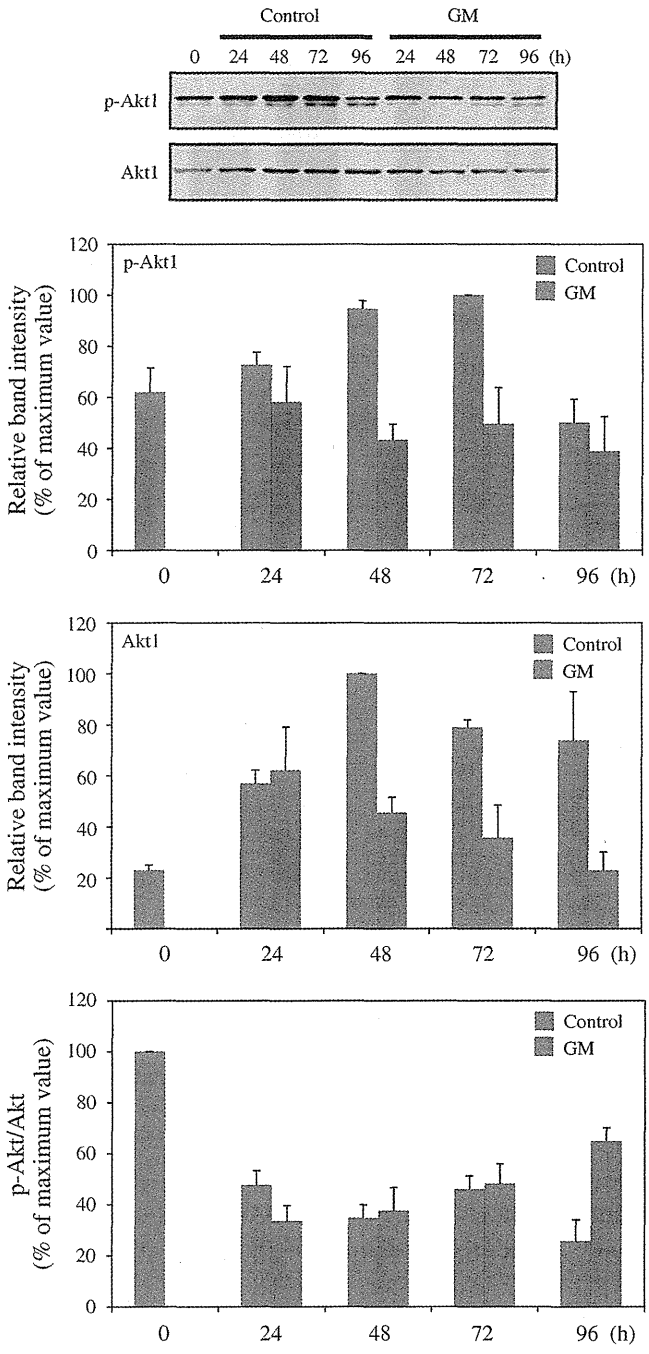


Fig. 5 Effects of geldanamycin treatment on levels of phosphorylated Akt1^{Ser473} and total Akt1 proteins during myogenic differentiation. Histograms represent the temporal changes in levels of phosphorylated Akt1^{Ser473}, total Akt1 proteins, or phosphorylated Akt1^{Ser473}/Akt1 during myogenic differentiation. The intensities of bands were measured and normalized to the maximum value observed during 4 days in culture. The data are the means ± SD (n = 3/time point)

that inhibition of HSP90 activity by geldanamycin is the cause for lower expression levels of its client proteins and myogenic regulatory factors in C2C12 cells. C2C12 cells were cultured in differentiation medium for 48 h and then treated with 17-AAG (250 nM) for 24 h. After treatment of 17-AAG, total cell lysates were prepared, and immunoblot analysis was performed with antibodies against the individual proteins. The levels of HIF-1α, MyoD, myogenin, and Akt1 were decreased in the 17-AAG-treated cells compared with in the untreated cells (Fig. 7).

Up-regulation of HSP90 coincides with increased expression of myogenic regulatory factors MyoD and myogenin during muscle degeneration/regeneration

It has been reported that up-regulation of HSP90 is observed in regenerating myofibers from patients with Duchenne muscular dystrophy [34], indicating that HSP90 expression may be regulated during myogenesis in vivo. Therefore, we monitored the temporal expression levels of HSP90, MyoD, and myogenin proteins during muscle regeneration. Muscle regeneration in gastrocnemius muscle was induced following injection of glycerol, which causes extensive and reproducible muscle necrotic injury. The myogenic differentiation is initiated within 2 days followed by extensive regeneration within 7-14 days after initiation of muscle injury [24]. Figure 8a shows the temporal changes in the amount of HSP90, MyoD, and myogenin proteins during muscle regeneration. A quantitative analysis showed that the amount of HSP90 protein increased to a peak at day 5, followed by a progressive decrease by day 14, and returned to the control level by day 28. The pattern of temporal changes in MyoD and myogenin protein levels was similar to that observed in the HSP90, exhibiting the same strong increase in day 5.

Localization of HSP90 protein in regenerating myofibers

To examine the cellular localization of the HSP90 protein during muscle degeneration/regeneration induced by injection of glycerol, immunocytochemical analysis was performed. Figure 8b shows HSP90 protein localization during muscle degeneration/regeneration. The HSP90 protein was mainly localized in nuclei of newly formed regenerating myofibers, a hallmark of regenerated myofibers, and also in mononuclear cells. The expression of HSP90 protein appeared to attenuate as myofibers, which further differentiated at later time points but was still detected in the regenerating myofibers.

Pharmacological inhibition of HSP90 activity affects muscle regeneration

To extend the results from cultured cells, we used muscle degeneration/regeneration model to examine the effect of geldanamycin on myogenic differentiation in vivo. Glycerol was injected into gastrocnemius muscles of control and

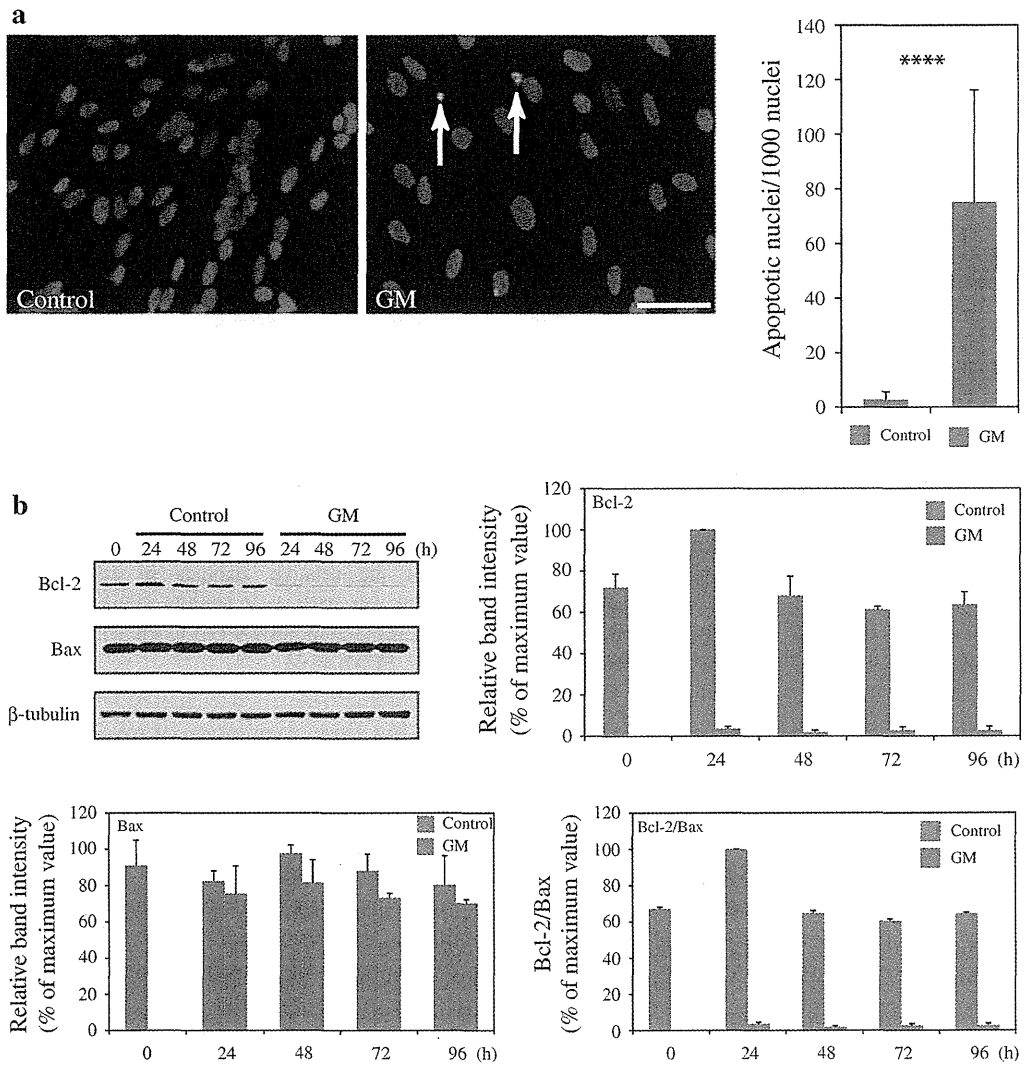


Fig. 6 Effects of geldanamycin treatment on apoptosis induction and expression of Bcl-2 and Bax proteins during myogenic differentiation. **a** The incidences of apoptotic nuclei (*arrows*) were detected by TUNEL method. Histogram represents the number of TUNEL-positive nuclei at 96 h. The data are the means ± SD of at least six independent experiments. **b** Histograms represent the temporal changes in the expression levels of each protein during myogenic differentiation. β-Tubulin was used as a loading control. The intensities of bands were measured and normalized to the maximum value observed during 4 days in culture. The data are the means ± SD ($n = 3$/time point). These were statistically significant differences compared to the control (Ct): $^{***}P < 0.001$

geldanamycin-treated mice. Ten days after initial injury, the muscles were removed, cryosectioned, and stained with hematoxylin and eosin. The control muscles contained numerous nascent myofibers with centrally located nuclei, whereas the skeletal muscles forced to regenerate in the presence of geldanamycin to inhibit the activity of HSP90 were of poor repair with small myofibers and increased connective tissues (Fig. 9a). A quantitative analysis showed that the area occupied by myofibers significantly decreased by 43% whereas the area occupied by non-myofibers was significantly increased by 284% in skeletal muscle when treated with geldanamycin (Fig. 9b). FCSA for regenerating myofibers was also significantly decreased by 62% (Fig. 9b). To elucidate the inhibitory effect of geldanamycin on muscle regeneration, we examined the expression levels of myogenic regulatory factors, MyoD and myogenin proteins. Total cellular protein extract was prepared at 7 days after induction of muscle injury, and immunoblot analysis was performed with antibodies against the individual proteins. The expression levels of MyoD and myogenin were decreased in the geldanamycin-treated mice compared with in the untreated mice (Fig. 9c).

Discussion

HSP90 plays some roles in cell differentiation and survival in a variety of cell types [35] including myogenic cells [8]. HSP90 activity has shown to be inhibited by geldanamycin as follows: Geldanamycin binds to conserved binding

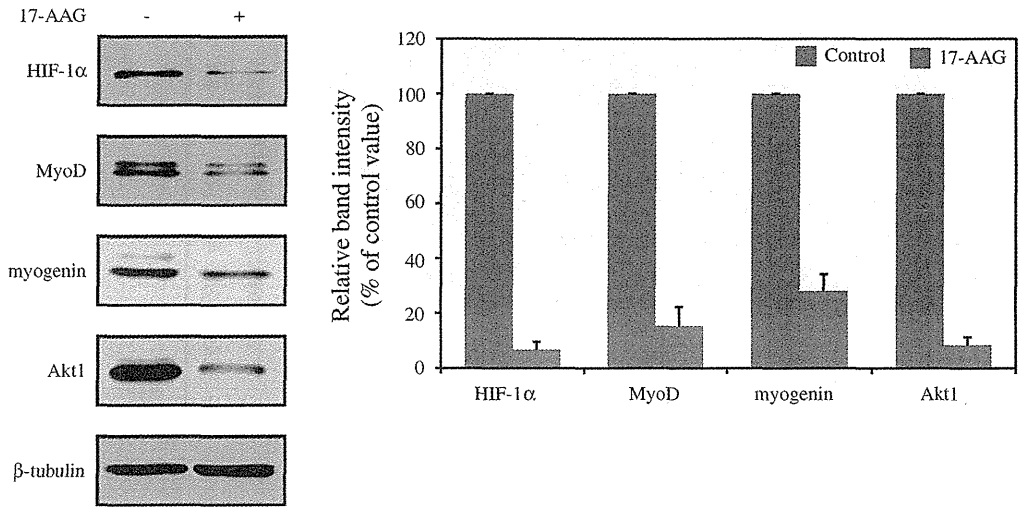


Fig. 7 Effects of geldanamycin derivative, 17-AAG, on HIF-1α, MyoD, myogenin, and Akt 1 proteins in the developing C2C12 cells. Cells were cultured with differentiation medium for 48 h and then treated with 17-AAG (250 nM) for 24 h. The expression levels of each protein were determined by immunoblot analysis with anti-HIF-1α, anti-MyoD, anti-myogenin, and anti-Akt1 antibodies. Histograms represent the relative changes in expression of each protein. β-Tubulin was used as a loading control. The intensities of bands were measured and normalized to the control value. The data are the means ± SD ($n = 3$)

pocket in the N-terminal domain of HSP90, inhibiting ATP binding and ATP-dependent release of the client proteins undergoing refolding from HSP90, leading to degradation of the client proteins by the ubiquitin-dependent proteasome pathway [36]. In this study, we examined the effect of pharmacological inhibition of HSP90 activity using geldanamycin on myogenic differentiation and cell survival in C2C12 cells. We demonstrated that myogenic differentiation was suppressed with decreased expression of myogenic regulatory factors, MyoD and myogenin, when the cells were cultured in the presence of geldanamycin. Phosphorylation levels of Akt and JNK, which are required for myogenic differentiation, were reduced in geldanamycin-treated cells compared with control cells. Confirming the results from cultured cells using in vivo model for muscle regeneration, mice treated with geldanamycin exhibited poor repair of muscle injury. Along with myogenic differentiation, apoptotic nuclei were increased in geldanamycin-treated cells compared with untreated cells with decreased expression of anti-apoptotic protein, Bcl-2. Together, our findings indicate that pharmacological inhibition of HSP90 activity may negatively modulate myogenic differentiation and may induce apoptotic cell death.

Our results extend observations from previous study [8], which has shown that geldanamycin blocks myotube formation, by providing more quantitative support for their data. Our result revealed a marked reduction in fusion capacity with only 3% nuclei being incorporated into the small myotubes in geldanamycin-treated cells. This observation could be achieved with a lower dose (0.01 μg/ml) compared with a dose (0.05 μg/ml) used in previous study [Yun 2005], and even at low dose, expression of myosin heavy chain could be suppressed in geldanamycin-treated cells. Geldanamycin-mediated inhibition of myogenic differentiation might be explained, at least in part, through down-regulation of myogenic regulatory factors to produce a skeletal muscle-specific expression pattern. Some possible mechanisms for this down-regulation can be envisioned. One possibility is that MyoD may be putative target protein for HSP90. HSP90 could rapidly convert MyoD from an inactive to an active conformation, whose conversion process involves a transient interaction between HSP90 and MyoD [37]. This may be supported by our observation that HSP90 protein was localized in the nuclei of regenerating myofibers, which expresses MyoD protein [38]. On the other hand, geldanamycin-induced inhibition of HSP90 activity has little effect on interaction between MyoD and HSP90, although the interaction of MyoD with the cochaperone adaptor protein, Cdc37, is disrupted by geldanamycin [8]. Thus, it is difficult to conclude that pharmacological inhibition of HSP90 activity is directly involved in decreased expression of MyoD observed in this study. Another possibility is that pharmacological inhibition of HSP90 activity indirectly influences on expression of myogenic regulatory factors. It has been reported that Akt activity is closely linked to the expression of myogenic regulatory factors. Targeted knockdown of Akt1 leads to depletion of MyoD and myogenin in C2C12 cells [39]. In addition, dominant-negative Akt decreases transcriptional activity of MyoD, and short hairpin RNA-mediated inhibition of Akt1 results in decreased myogenin promoter activity [40]. Thus, down-regulation of myogenic regulatory factors observed in geldanamycin-treated cells may be due to depletion of Akt after induction of myogenic differentiation.

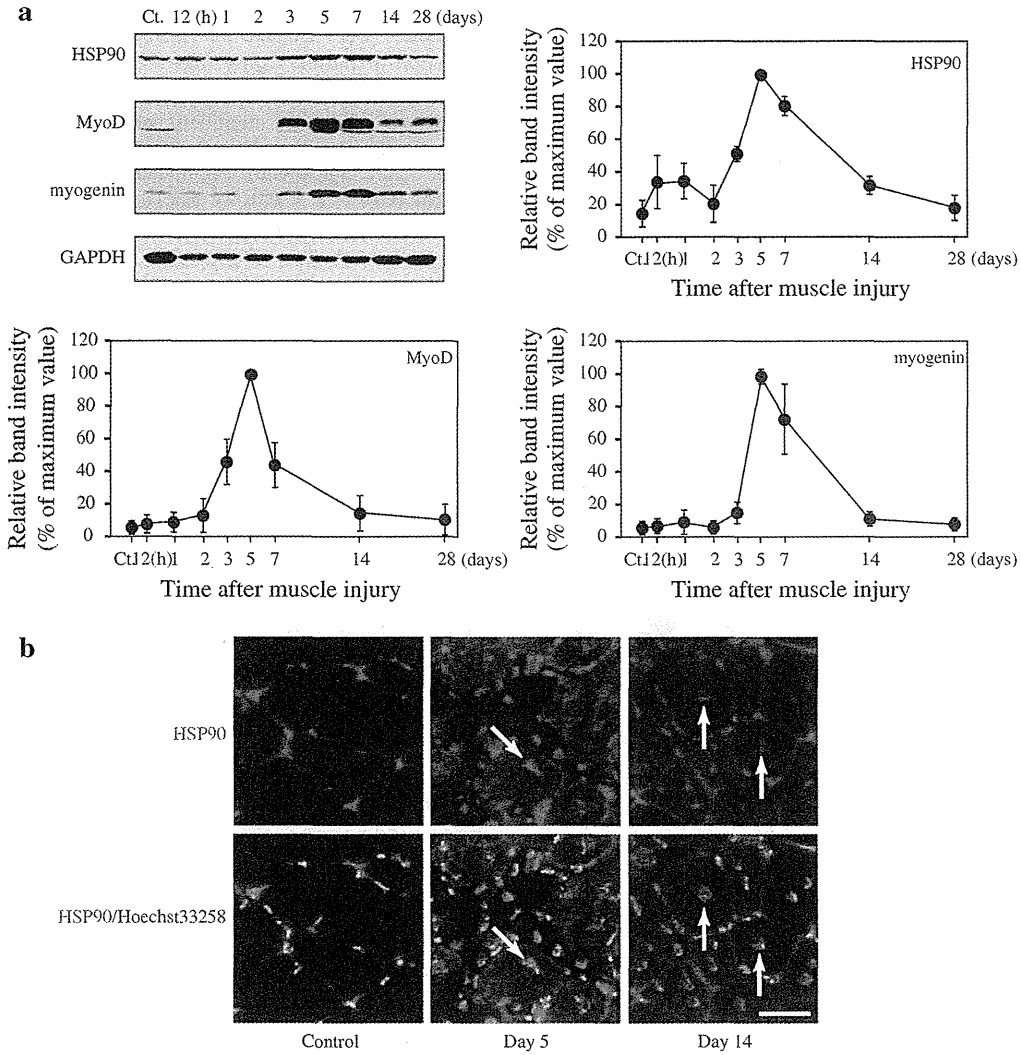
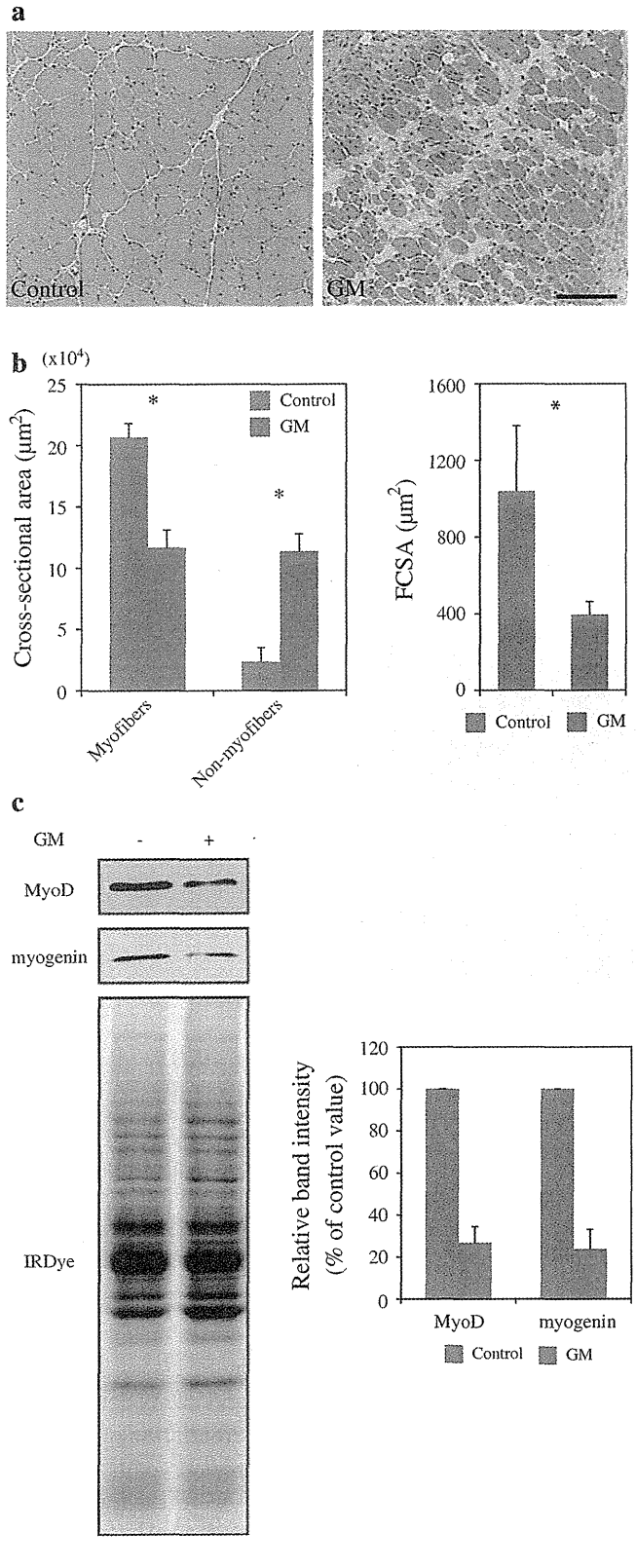


Fig. 8 Temporal changes in expression of HSP90, MyoD, and myogenin proteins during muscle degeneration/regeneration. **a** Tissues were isolated for immunoblot analysis at various time points [Control (Ct), 12 h, 1, 2, 3, 5, 7, 14, or 28 days]. Line graphs represent the temporal changes in the expression levels of each protein during myogenic differentiation. GAPDH was used as a loading control. The intensities of bands were measured and normalized to the maximum value observed during muscle degeneration/regeneration. Because of the extensive myofiber necrosis at early time points of muscle degeneration/regeneration, the amount of GAPDH was not equal across all the time points, as expected. The data are the means ± SD (n = 3/time point). **b** HSP90 protein localization on day 5 and day 14 after muscle injury. The fixed tissues were stained with anti-HSP90 antibody, and nuclei were counterstained with Hoechst 33258. Arrows indicate a nuclear localization of HSP90 protein in regenerating myofibers. All experiments were performed at least three times. *Scale bar* = 50 μm

Consistent with previous study [8], inhibition of HSP90 activity by geldanamycin resulted in progressive loss of Akt protein after induction of myogenic differentiation, likely through the ubiquitin proteasome pathway. Indeed, proteasome inhibitor, lactacystin, partially protects Akt protein from geldanamycin-mediated loss [41]. It has been shown that geldanamycin causes newly synthesized Akt protein to be degraded rapidly but has little effect on mature Akt protein in C2C12 cells [8]. Thus, geldanamycin-mediated depletion of Akt1 observed in this study appears to be due to the inability of the cells to mature newly synthesized Akt1 protein into a stable conformation.

In this study, the phosphorylation levels of Akt1^{Ser473} were increased maximally at 72 h and were decreased at 96 h after induction of myogenic differentiation in untreated cells, whereas in geldanamycin-treated cells, reduced phosphorylation levels of Akt1^{Ser473} were continued. This suggests that phosphorylation levels of Akt1^{Ser473} may be temporally regulated to sufficient to differentiate into myoblasts and fuse to form myofibers. Akt1 is phosphorylated at two key regulatory sites, Thr308 and Ser473, by 3-phosphoinositide-dependent kinase-1 (PDK1) [42] and by mammalian target of rapamycin complex 2 (mTORC2) [43], respectively. Since Thr308 phosphorylation is necessary for



◀ **Fig. 9** Effects of geldanamycin treatment on muscle regeneration. **a** Tissues were isolated for histochemical analysis 10 days after initiation of muscle injury, fixed, sectioned, and then stained with hematoxylin and eosin. *Scale bar* = 100 μm. **b** Histograms represent the areas occupied by myofibers and non-myofibers, or FCSA. The data are the means ± SD of at least six independent experiments. These were statistically significant differences compared to the control (Ct): $^{**}P < 0.01$. **c** The expression levels of each protein were determined by immunoblot analysis with anti-MyoD and anti-myogenin antibodies. Histograms represent the relative changes in expression of each protein at day 7 after induction of muscle injury. Membranes were stained with IRDye to verify equal loading. The intensities of bands were measured and normalized to the control value. The data are the means ± SD ($n = 3$)

activation of Akt1 and Ser473 phosphorylation is only required for maximal activity [44], alterations in PDK1/mTORC2 activities could undoubtedly contribute to the decline of Akt1 activity observed in geldanamycin-treated cells. Since PDK1 has been shown to be HSP90 client protein [45], geldanamycin-mediated depletion of PDK1 may result in reduced phosphorylation levels of Akt1^{Thr308}. However, it is unlikely that reduced phosphorylation levels of Akt1^{Ser473} observed in this study may be due to reduced mTORC2 activity, since the essential mTORC2 component, rictor, which is required for phosphorylation of Ser473 in cells [46], has not been reported to be HSP90 client protein. In addition, Akt activity is also modulated through the regulation of dephosphorylation. Protein phosphatase 2A (PP2A) has been proposed to be the most likely candidate for regulating the rate of Akt dephosphorylation [47]. It has been reported that HSP90 binding to Akt protects Akt protein from PP2A-mediated dephosphorylation, whereas detachment of Akt from HSP90 promotes the PP2A-mediated dephosphorylation of Akt [47]. Thus, reduced phosphorylation of Akt1^{Sre473} observed in geldanamycin-treated cells may be due to imbalance between phosphorylation and dephosphorylation by mTORC2 and PP2A.

Unexpectedly, we observed transient up-regulation of MyoD and myogenin in geldanamycin-treated cells at 24 h after induction of myogenic differentiation. However, Yun et al. report that MyoD expression remains unchanged and myogenin could not be detected in geldanamycin-treated cells at 20 h after induction of myogenic differentiation. This is in disagreement with our results. While it is difficult to explain these contradictory data, one possibility may be changes in Akt1 activity in geldanamycin-treated cells. It is interesting to note that short-term (30 min) treatment of differentiating cells with geldanamycin does not cause any decrease in Akt and increases the levels of Akt phosphorylation at Ser^{473} but not those at Thr^{308}, which correlates with an increased phosphorylation levels of glycogen synthase kinase-3β [41]. Akt1 activity correlates to MyoD and myogenin expression during myogenic differentiation. Thus, we hypothesized that activation of Akt1 by phosphorylation at Ser^{473} may occur immediately after C2C12 cells were treated with geldanamycin, resulting in inducing MyoD and myogenin at the early stage of myogenic differentiation. Another possibility is that geldanamycin can have some side effects more or less independent of HSP90.

However, as aforementioned, the concentration used in this study is lower than that used in the previous study [8]. Thus, further study may be required to elucidate the effects of geldanamycin on the expression of myogenic regulatory factors at the early stage of myogenic differentiation.

A potentially important observation that emerged from this study was that the phosphorylated levels of JNK were decreased when the C2C12 cells were treated with geldanamycin. This study is, to the best of our knowledge, the first to deal with the potential regulation of JNK in C2C12 cells using geldanamycin. JNK has been involved in controlling diverse cellular functions, including cell proliferation, differentiation, and survival [48]. Basal JNK activity appears to be required for myogenic differentiation and cell survival. Inhibition of JNK activities by JNK inhibitor II drastically inhibits myogenic differentiation and increases apoptotic nuclei in L6E9 cells [20]. Activation of JNK is mediated by a MAP kinase module, MAPKKK (MAP3K) → MAPKK (MAP2K) → MAPK through sequential protein phosphorylation. The mixed-lineage kinases (MLKs) function as MAP3K to activate the JNK pathway [49]. MLK3 activates the JNK pathway through phosphorylation and activation of MAPK kinase 4 (MKK4) [50] and MKK7 [51]. Thus, we hypothesized that sequential activation pathway may be partially impaired, resulting in reduced phosphorylation levels of JNK in geldanamycin-treated cells. This hypothesis may be supported in part by the observation that geldanamycin decreases the endogenous level of MLK3 and abolishes TNF-mediated activation of MLK3 and JNK without affecting MKK4 and MKK7 in vitro [16]. Thus, reduced phosphorylated JNK, which is regulated possibly at the MAP3K level, may contribute to geldanamycin-mediated inhibition of myogenic differentiation and cell survival.

We could fail to observe any changes in the phosphorylation levels of ERK1/2 and p38MAPK in geldanamycin-treated cells. The exact mechanism by which geldanamycin has no effect on phosphorylation levels of p38MAPK and ERK1/2 remains unclear. The response of MAPKs to geldanamycin appears to be different according to cell type. Geldanamycin transiently activates ERK2 activity at 8-9 h after treatment but does not JNK1 activity in neuroblastoma cell line [21]. The phosphorylation levels of ERK1/2 remain unchanged at 16 h after treatment in embryonic fibroblast cell line [15]. Geldanamycin derivative, 17-AAG, also reduces the phosphorylation levels of ERK1/2, p38MAPK, and JNK at 24 h after treatment in colon cancer cell lines [52]. These results indicate that the response of MAPKs to geldanamycin may be cell-type specific, depending on the dose and time of the treatment.

It has been demonstrated that the geldanamycin-treated cells for 24 h undergo apoptosis using a marker for the initial stage of apoptosis, cleaved poly(adenosine diphosphate–ribose ribose) polymerase [8]. We also showed that apoptotic nuclei were increased in geldanamycin-treated cells using a relatively late marker, DNA fragmentation as assessed by TUNEL. To gain insight into apoptotic response of C2C12 cells to geldanamycin, we focused on the Bcl-2 family, which is the best-characterized protein family involved in the regulation of apoptotic cell death, consisting of anti-apoptotic and pro-apoptotic members. We observed decreased expression levels of Bcl-2 protein but not those of Bax in the geldanamycin-treated cells. Bcl-2 localized in the mitochondrial membrane prevents the apoptosis-associated release of cytochrome *c* [53, 54] and apoptosis-inducing factor (AIF) [55] from the mitochondrial inter-membrane space into the cytoplasm. HSP90β has been shown to associate with Bcl-2 in mast cells. Depletion of HSP90β with a siRNA or inhibition of HSP90 with geldanamycin inhibits HSP90 interaction with Bcl-2 [22]. Thus, geldanamycin may conduce to unbalanced Bcl-2/Bax ratio in C2C12 cells, resulting in release of cytochrome c, caspase activation, and/or AIF, chromatin condensation, and DNA degradation, eventually leading to apoptosis. We observed that the majority of TUNEL-positive cells were mononuclear cells treated with geldanamycin. When proliferating myoblasts are induced to differentiate by deprivation of serum in the medium, a significant proportion of cells escape from terminal differentiation. The undifferentiated cells refer to as reserve cells [56]. It has been reported that Bcl-2 is expressed at the high levels in the reserve cells whereas it is expressed at very low levels in the myotubes [57], suggesting that Bcl-2 becomes the molecular marker of reserve cells. Although the physiological role of Bcl-2 expressed in the reserve cells is not fully understood, Bcl-2-positive cells resist apoptosis and are at an early stage of a process leading from muscle progenitor cell to myotube [58]. Thus, reduced levels of Bcl-2 observed in this study may reflect the loss of reserve cells, which would be eliminated possibly through apoptosis-dependent pathway.

Although HSP90 has been shown to be expressed in regenerating myofibers, it remains unknown whether HSP90 contributes to myogenesis in vivo. Given that HSP90 is involved in myogenic differentiation, expression of myogenic regulatory factors and HSP90 would be regulated in a coordinated manner during muscle regeneration. We showed that MyoD, myogenin, and HSP90 were simultaneously up-regulated at day 5 after induction of muscle injury. In addition, HSP90 was localized in nuclei of newly formed myofibers (day 5) and still present in the nuclei of regenerating myofibers at the later stage of muscle regeneration (day 14). It has been demonstrated that MyoD and myogenin are coexpressed in newly formed myofibers and they persists in the nuclei of regenerating myofibers for at least 2 weeks [38]. Thus, MyoD, myogenin, and HSP90 may be coexpressed in the regenerating myofibers. MyoD appears to induce terminal cell cycle

arrest during myogenic differentiation by increasing the expression of cyclin-dependent kinase inhibitor, $p21^{WAF1/CIP1}$ [59]. The myogenic regulatory factors activate transcription of muscle-specific genes by binding, upon heterodimerization with ubiquitous E proteins [60], the E-box consensus sequence in skeletal muscle gene promoters and enhancers [61]. Considering their role in myogenesis, elevated levels of HSP90 in regenerating myofibers may reflect an increased demand for its basal functions during cell cycle growth arrest and/or the progressive expression of the muscle phenotype.

It is likely that geldanamycin and 17-AAG may have positive and negative effects on muscle regeneration in vivo. Kayani et al. report that 17-AAG-mediated up-regulation of HSP70 results in improved recovery of force generation in skeletal muscles of old mice following lengthening contraction-induced damage [62]. Similar results have been shown in HSP70 transgenic mice [63]. Indeed, HSP70 overexpression inhibits NF-κB transcriptional activity [64], which acts as a negative regulator of myogenic differentiation [65]. On the other hand, we showed that pharmacological inhibition of HSP90 activity impaired muscle regeneration with decreased expression levels of MyoD and myogenin in vivo. Geldanamycin treatment resulted in the decreased size of regenerating myofibers, which could partially reflect the results from cultured cells. We cannot exclude the possibility that geldanamycin may have unexpected and undesirable side effects more or less independent of HSP90. Thus, we examined the toxic side effects of geldanamycin throughout experimental period. No pathological symptoms were observed by visual inspection, and no decreases in body weight were observed at a concentration used in this study (data not shown). Thus, we believe that its side effects are minimal. Besides myofiber regeneration, pharmacological inhibition of HSP90 activity might have an impact on neuromuscular junction (NMJ) formation, which is also essential aspect of the muscle regeneration process [30]. It has been reported that HSP90β plays an important role in NMJ formation in vivo by injecting 17-AAG intraperitoneally into embryos in utero at E14.5 [66], a time when the NMJ starts to form [67]. Previous study and our results indicate that HSP90 may be involved in myogenesis as well as NMJ formation in vivo. Although geldanamycin and its derivatives may have therapeutic benefit in the recovery following muscle damage, they have also the potential adverse effect on the viability of myogenic cells through their ability to impair the function of multiple HSP90-dependent signal pathways that are critical for cell differentiation and survival.

In conclusion, pharmacological inhibition of HSP90 activity led to depletion of signal transduction proteins whose are necessary for myogenic differentiation and cell survival. As HSP90 has been found to be either overexpressed or constitutively more active in cancer cells [68], HSP90 is an emerging target in cancer treatment due to its important role in maintaining transformation and in increasing the survival and growth of cancer cells [69]. 17-AAG, which has similar antitumor activity to that of geldanamycin with less toxicity [70], is currently being tested in ongoing phase 1 and phase 2 clinical trials [71]. Our results suggest that geldanamycin showed inhibitory effects on myogenesis in vivo and in vitro, which provides information that myogenic cells were sensitive to HSP90 inhibition, depending on experimental conditions.

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Mitochondrial adaptations in skeletal muscle to hindlimb unloading

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Abstract To gain insight into the regulation of mitochondrial adaptations to hindlimb unloading (HU), the activity of mitochondrial enzymes and the expression of nuclear-encoded genes which control mitochondrial properties in mouse gastrocnemius muscle were investigated. Biochemical and enzyme histochemical analysis showed that subsarcolemmal mitochondria were lost largely than intermyofibrillar mitochondria after HU. Gene expression analysis revealed disturbed or diminished gene expression patterns. The three main results of this analysis are as follows. First, in contrast to peroxisome proliferator-activated receptor γ coactivator 1 β (PGC-1β) and PGC-1-related coactivator, which were down-regulated by HU, PGC-1α was up-regulated concomitant with decreased expression of its DNA binding transcription factors, PPARα, and estrogen-related receptor α (ERRα). Moreover, there was no alteration in expression of nuclear respiratory factor 1, but its downstream target gene, mitochondrial transcription factor A, was down-regulated. Second, both mitofusin 2 and fission 1, which control mitochondrial morphology, were down-regulated. Third, ATP-dependent Lon protease, which participates in mitochondrial-protein degradation, was also down-regulated. These findings suggest that HU may induce uncoordinated expression of PGC-1 family coactivators and DNA binding transcription factors, resulting in reducing ability of mitochondrial biogenesis. Furthermore, down-regulation of mitochondrial morphology-related genes associated with HU may be also involved in alterations in intracellular mitochondrial distribution.

Keywords Adaptation · Atrophy · Hindlimb unloading · Mitochondria · Skeletal muscle

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Introduction

Mitochondria play important roles in energy homeostasis, metabolism, signaling, and apoptosis [1]. The abundance, morphology, and functional properties of mitochondria are dynamically regulated in response to alterations in neuromuscular activity. Basically, physical activity (e.g., exercise, endurance training, and interval training) promotes mitochondrial biogenesis, [2] whereas disuse (e.g., denervation, immobilization, spaceflight, and hindlimb unloading) discourages mitochondrial biogenesis [3–5].

A model of hindlimb unloading (HU) of a rodent is frequently used to simulate and study neuromuscular perturbations occurring in a real microgravity environment during spaceflights [6]. This earth-based model of microgravity is characterized by reduction of motor activity and lack of load bearing [7]. The major modifications concerning HU are muscle atrophy [4] and slow-to-fast fiber-type switching [7]. In addition, experimental evidence for mitochondrial adaptations to HU is accumulating; for example, mitochondrial fragmentation [8], decreased mitochondrial enzyme activity [9–14], and reduced mitochondrial oxygen consumption [9] have been reported. However, these adaptive mechanisms concerning HU remain to be elucidated. Understanding these adaptive

mechanisms may extend our knowledge of skeletal muscle plasticity and provide important insights into both space-flight- and earth-based health problems.

The adaptive mechanisms may be associated with transcriptional alterations in expression of mitochondrial biogenesis-related and other mitochondria-related genes. Mitochondrial biogenesis is a complex biological process that requires the system to import and incorporate proteins and lipids into the existing mitochondrial reticulum as well as to replicate mitochondrial DNA (mtDNA) [1]. The transcriptional mechanism that controls mitochondrial biogenesis is a complex network. The peroxisome proliferator-activated receptor, γ, coactivator 1 (PGC-1) family of transcriptional coactivators interact with multiple DNA binding transcription factors to coordinate the regulation of multiple mitochondrial genes [1]. PGC-1α coactivates expression of estrogen-related receptor α (ERRα), which activates expression of peroxisome proliferator-activated receptor α (PPARα), nuclear respiratory factor 1 (NRF-1), and NRF-2 [15]. NRF-1 activates expression of oxidative phosphorylation components, mitochondrial transporters, and mitochondrial ribosomal proteins [1]. In addition, NRF-1 regulates expression of mitochondrial transcription factor A (TFAM), contributing to mtDNA replication and transcription [16]. Apart from mitochondrial biogenesis, a recent report has highlighted the importance of mitochondrial dynamics in cell and animal physiology. Mitochondria constantly fuse and divide, and an imbalance of these two processes dramatically alters overall mitochondrial morphology and function [17]. Mitochondrial fission is driven by dynamin 1-like and fission 1, while mitochondrial fusion is controlled by mitofusins and OPA 1 [18]. Moreover, ATP-dependent Lon protease is known to catalyze the degradation of oxidatively modified matrix proteins [19]. These nuclear-encoded genes which control mitochondrial properties may therefore be key contributors to mitochondrial adaptations to HU.

In this study, we hypothesize that mitochondrial adaptations to HU are due, at least in part, to transcriptional alterations in expression of nuclear-encoded genes which control mitochondrial properties. To test this hypothesis, we investigated the activity of mitochondrial enzymes and the expression of nuclear-encoded genes involved in mitochondrial biogenesis, mitochondrial morphogenesis, and mitochondrial-protein degradation in mouse gastrocnemius muscle.

Materials and methods

Animals

Female seven-week-old CD1 mice (Clea Japan, Meguro, Tokyo) were used and housed in the animal care facility under a 12-h light/12-h dark cycle at room temperature (23 ± 2°C) and 55 ± 5% humidity. Mice were matched by body weight and then assigned to one of two groups as follows: ground-based control ($n = 12$) or HU ($n = 12$). The mice were procured after approval for this study from The University of Tokyo Animal Ethics Committee.

Preparation of mitochondria population

Two mitochondrial fractions, termed subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, were isolated from skeletal muscle as previously reported [20]. Briefly, the skeletal muscle (the deep-red region of the gastrocnemius) was removed and immediately placed in ice-cold isolation buffer (IB). The muscles were freed of fat and connective tissue, transferred into fresh IB, and weighed. All further procedures were carried out at 0–5°C. The muscle sample was next minced with triple scissors in IB. The muscle sample was homogenized by using a tissue homogenizer for 10 s. The homogenate was centrifuged at 800*g* for 10 min, and the resulting precipitate was subsequently used for the preparation of the IMF mitochondria. The final SS mitochondrial pellet was suspended in IB. The pellet from the 800*g* centrifugation contained primarily intact material with some remaining SS mitochondria. This pellet was then washed, and the IMF mitochondria were liberated with nagarse incubation. After two washes were performed, the final IMF mitochondrial pellet was suspended in IB. The final mitochondrial protein concentration was determined by a micro bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard.

Procedure for hindlimb unweighting

A rat-hindlimb-suspension model, originally described in [21], was modified for use with mice [22]. Briefly, each mouse was weighed and anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body weight). We wrapped bandages (Nichiban, Bunkyo, Tokyo) around the tail of the mice. After the mouse had recovered from the anesthetic, a swivel hook was placed through the bandage distal to the tip of the tail. This method allows the animals full 360° rotation as well as access to food and water without allowing the hindlimbs to contact the cage floor or walls. HU was continued for 7 days. All the procedures in the animal experiments were carried out in accordance with the guidelines presented in the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, published by the Physiological Society of Japan.

Enzyme activity assay

The activity of citrate synthase (CS) was determined spectrophotometrically according to Srere [23]. Briefly, skeletal muscle (the deep-red region of the gastrocnemius) was homogenized in 175 mM KCl, 10 mM GSH, and 2 mM EDTA at pH 7.4. The homogenate thus obtained was frozen and thawed four times and mixed thoroughly before enzymatic measurements were performed. The principle of the assay is to initiate the reaction of acetyl-CoA with oxaloacetate and the link the release of free CoA-SH to a colorimetric reagent, 5,5-dithiobis-(2-nitrobenzoate) (DTNB). The absorbance of the reaction mixtures was monitored at wavelength of 405 nm at 15 s intervals for a period of 3 min. The activity of 3-hydroxyacyl-CoA dehydrogenase (3-HAD) was assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH in the presence of acetoacetyl-CoA as described [24]. The absorbance of the reaction mixtures was monitored at 15 s intervals for a period of 5 min. The enzyme activities were normalized in regard to wet weight of skeletal muscle.

Enzyme histochemical analysis

To examine mitochondrial location in a myofiber, succinate dehydrogenase (SDH) staining was carried out according to Nachlas et al. [25] with minor modification. Briefly, frozen transverse sections from the mid-belly region of the gastrocnemius muscles were incubated for 10 min at 37°C in a medium (0.9 mM 1-methoxyphenazine methylsulfate, 1.5 mM nitro blue tetrazolium, 5.6 mM EDTA, and 48 mM succinate disodium salt at pH 7.6) in a 0.1 M phosphate buffer, rinsed for 5 min with deionized H_2O, dehydrated for 5 min within a myofiber in 50% acetone, and then air-dried. The fiber cross-sectional area (FCSA) and length of SDH activity wit were measured by using ImageJ software (ver. 1.42, http://rsb.info.nih.gov/ij/).

Gene expression analysis

Total RNA was prepared with TRI reagent (Molecular Research Center, Cincinnati, OH). The DNase-treated total RNA was converted to cDNA by using a first-strand cDNA synthesis system for quantitative RT-PCR (Marligen, Biosciences, Ijamsville, MD). The cDNA samples were aliquoted and stored at −80°C. Real-time PCR was carried out using an Opticon™ DNA engine (MJ Research, Waltham, MA) according to the manufacturer's instructions. The reactions employed gene-specific primers for citrate synthase, mitofusin 2, fission1, Lon protease (Takara Bio Inc., Otsu, Shiga), succinate dehydrogenase [26], medium-chain acyl-coenzyme A dehydrogenase (MCAD) [27], peroxisome proliferator-activated receptor, γ, coactivator 1α (PGC-1α), peroxisome proliferator-activated receptor, γ, coactivator 1β (PGC-1β) [28], peroxisome proliferator-activated receptor, γ, coactivator-related 1 (PRC), PPARα [29], mitochondrial transcription factor A (TFAM), mitochondrial single-stranded DNA binding protein 1 (mtSSB) [30], NRF-1, NRF-2 [31], ERRα [32], and manganese superoxide dismutase (MnSOD) [33]. PCR thermal-cycle conditions were optimized to achieve a single ethidium bromide-stained band following electrophoresis on a 2% agarose gel. Differences in gene expression were calculated relative to the expression of a housekeeping gene by comparison with a standard curve. To identify the appropriate housekeeping gene, we investigated several housekeeping genes including 18S ribosomal RNA, glyceraldehyde-3-phosphate dehydrogenase, β-actin, and cyclophilin. We selected cyclophilin as the housekeeping gene because its expression levels remain unchanged in hindlimb-unloaded muscle relative to control muscle. Cyclophilin was found to be appropriate for normalizing the signal by comparing the differences in raw threshold cycle values (i.e., the number of amplification cycles at which the signal is detected above the background and is in the exponential phase). A standard curve was constructed from serially diluted cDNA of gastrocnemius muscle. Each sample was normalized by its cyclophilin content. The final results were expressed as a relative fold change compared to that of control animals.

Statistics

Data are means ± SEM. For analysis between the control and HU cases, Student's t test was used to determine significance. The level of significance was set at $P < 0.05$.

Results

To assess whether the mouse model mimics the rat model previously reported, the degree of muscle atrophy was estimated, and the expression of atrogin-1 mRNA, muscle-specific ubiquitin-ligase, which is known to mediate rodent muscle atrophy, was investigated. Myofibers from hindlimb-unloaded mice were pathologically atrophied in reference to those from control mice. Significant decreases in the FCSA of two types of myofiber (52% for SDH^{High} and 51% for SDH^{Low}) were observed after 7 days of HU. Moreover, atrogin-1 mRNA transcript was strongly induced in the HU case compared to the control case (Fig. 1).

Subsarcolemmal mitochondria were degraded more rapidly than IMF mitochondria during HU [8]. Therefore, we isolated SS and IMF mitochondria from control and hindlimb-unloaded mice. As expected, SS mitochondria

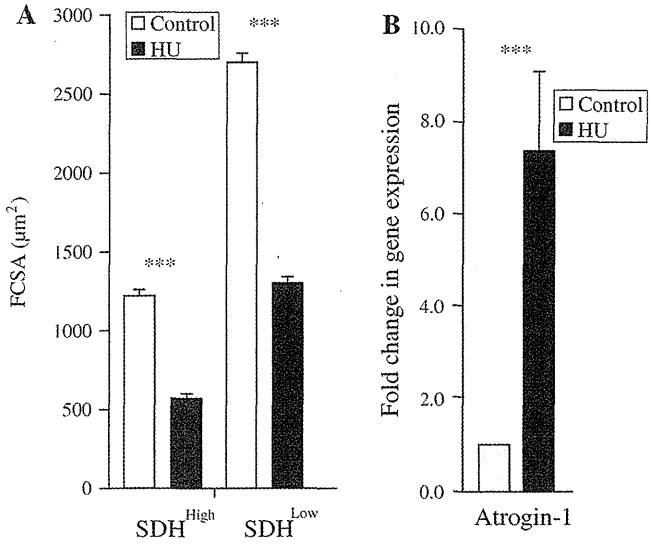

Fig. 1 Effects of HU on myofiber size and mRNA expression levels of atrogin-1, a muscle-specific ubiquitin-ligase required for muscle atrophy. **a** After 7 days of HU, muscle tissues were cryosectioned and reacted for SDH activity. Myofibers were divided into two groups: SDH^{High} (dark stain) and SDH^{Low} (light stain). FCSA was measured with the image-analysis system, calibrated to transform the number of pixels (viewed on a computer monitor) into micrometers. **b** To investigate mRNA expression levels between control and HU, total RNA was isolated from muscle tissues and relative gene expression was quantified by real-time PCR. Cyclophilin was used internal control. The data are means ± SEM ($n = 6$). Statistically significant differences compared to control: $***P < 0.001$

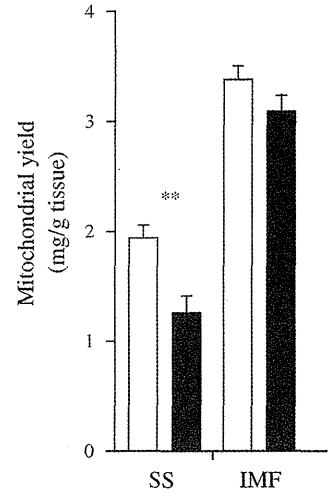

Fig. 2 Effects of HU on quantitative alteration in mitochondria in SS and IMF regions of muscle fibers. SS and IMF mitochondrial fractions were isolated from control and hindlimb-unloaded mice by differential centrifugation and digestion techniques. Mitochondrial protein content was determined using micro BCA assay and the yield was expressed as mg/g tissue weight. The data are means ± SEM ($n = 6$). Statistically significant differences compared to control: $**P < 0.01$

were significantly decreased in the HU case compared to that in the control case. Although IMF mitochondria also showed a tendency to decrease after HU, there was no significant difference between groups (Fig. 2).

Because HU decreases the activity of mitochondrial enzymes, the activity of CS was measured. The activity was significantly decreased in the HU case compared to that in the control case. It has been reported that HU suppressed gene expression of enzyme in fatty-acid oxidation [34]. Therefore, we measured the activity of 3-HAD, a key enzyme in muscle fatty-acid oxidation. The activity was significantly decreased in the HU case compared to that in the control case. CS and MCAD mRNA transcripts levels were significantly decreased in the HU case compared to that in the control case.

To examine whether HU affects the location-specific differences in mitochondrial enzyme activity, an enzyme histochemical SDH staining on cryosections was carried out. SDH activity in SS and IMF regions in the control and hindlimb-unloaded mice was observed (Fig. 3a). The area of SS SDH activity in SDH^{High} myofibers was measured. The SS SDH activity was significantly decreased in the HU case compared to the control case, whereas ratio of SDH activity area to FCSA was insignificantly decreased slightly (Fig. 3b). Furthermore, the length of SS SDH activity in SDH^{High} myofiber was measured. This measurement shows that the length was significantly decreased in the HU case compared to the control case (Fig. 3c). Next, the area of IMF SDH activity in SDH^{High} myofibers was measured. The SDH activity was significantly decreased in the HU case compared to the control case, whereas the ratio of SDH activity area to FCSA was significantly increased (Fig. 3d). In addition, SDH mRNA transcript level was significantly decreased in the HU case compared to the control case (Fig. 3e).

To examine the effects of HU on expression of mitochondrial biogenesis-related genes, RNA samples from the gastrocnemius of control and hindlimb-unloaded mice were subjected to cDNA synthesis and real-time PCR. The PGC-1 family, including PGC-1α, -1β, and PRC, is known to play a central role in mitochondrial biogenesis. PGC-1α mRNA transcript level was significantly increased in the HU case compared to the control case, whereas PGC-1β and PRC mRNA transcript levels were significantly decreased. The transcript level of DNA binding transcription factors, which interact with PGC-1 coregulators, was investigated. Both PPARα and ERRα mRNA transcripts levels were significantly decreased in the HU case compared to the control case. In contrast, NRF-1 mRNA transcript level was insignificantly decreased in the HU case compared to the control case, whereas NRF-2 mRNA transcript level was significantly increased. TFAM and mtSSB function as a key regulator of mammalian mtDNA maintenance. TFAM mRNA transcript level was significantly decreased in the HU case compared to the control

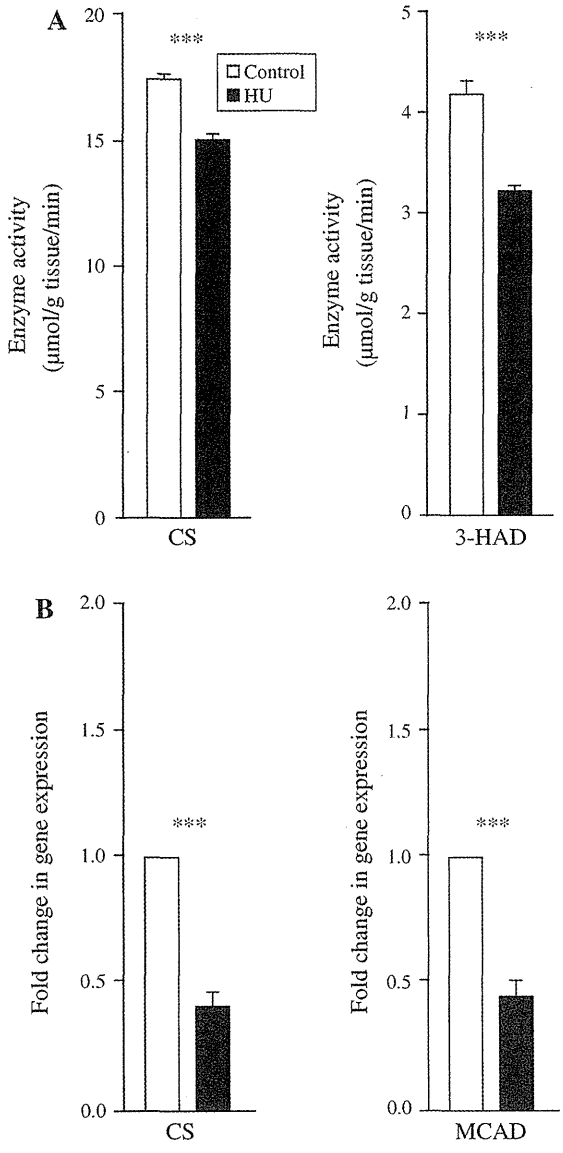

Fig. 3 Effects of HU on activities of mitochondrial enzymes and mRNA expression levels of mitochondrial enzymes. **a** The tissue homogenates were subjected to freeze-thaw cycles, centrifuged, and assayed for enzyme activities. The enzyme activity was normalized for wet weight of skeletal muscle. **b** Total RNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. The transcript levels were compared to control mice. The data are means ± SEM ($n = 6$). Statistically significant differences compared to control: $^{***}P < 0.001$

case, whereas mtSSB mRNA transcript level was significantly increased (Fig. 4).

The transcript level of two GTPase proteins that act in opposing fusion and fission pathways for maintaining the dynamic of tubular mitochondrial networks were investigated. Both mitofusin 2 mRNA and fission 1 mRNA transcripts levels were significantly decreased in the HU case compared to the control case. ATP-dependent Lon protease mRNA transcript level, which is implicated in mitochondrial protein degradation, was significantly decreased in the HU case compared to the control case (Fig. 5).

Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle [35]. MnSOD (localized in the mitochondrial matrix) provides a major defense against oxidative damage by reactive oxygen species (ROS). MnSOD mRNA transcript level remained unchanged in the HU case compared to the control case (Fig. 6).

Discussion

Mammalian mitochondria exhibit a remarkable capacity to adapt to physiological and pathophysiological demands. The processes by which these adaptations occur are thought to be largely achieved at the level of transcriptional regulation. Accordingly, this study focused on the activity of mitochondrial enzymes and the expression of nuclear-encoded genes involved in mitochondrial biogenesis, mitochondrial morphogenesis, and mitochondrial-protein degradation after HU. Our findings provide the insight that mitochondrial adaptations to HU may be associated with distorted gene expression, resulting in changes in the abundance, morphology, and functional properties of mitochondria.

The modified hindlimb suspension model was chosen because it is considered less stressful on the animal than the harness method. Differences in body weights throughout the experimental period can influence interpretation of the experimental results [6]. In our experiment, mice from the hindlimb-unloaded group showed no significant difference in body weight compared with the control group (data not shown), suggesting that the stress effects that occurred during 7 days of HU were minimal. To verify the efficiency of the HU, the changes in myofiber size after 7 days of HU were investigated. The degree of change in our experiment was greater than that in previously reported ones [36, 37]. This difference may be attributed largely to differences between the experimentation set-ups used in the laboratories concerned. The expression of atrogin-1, which is generally recognized as a molecular marker for muscle atrophy, was investigated. A strong up-regulation of atrogin-1 after HU, which is in line with previous reports [38–40], was observed. HU reduced the activity of mitochondrial enzymes as previously reported [9–14]. These findings demonstrate the reliability of our HU model.

Mitochondria that are clustered in proximity to the sarcolemma are termed SS mitochondria, and those embedded among myofibrils are called IMF mitochondria [41]. In this study, SS mitochondria were lost largely than IMF mitochondria after HU. This finding is in keeping with the previous report that the absolute volumes of SS and

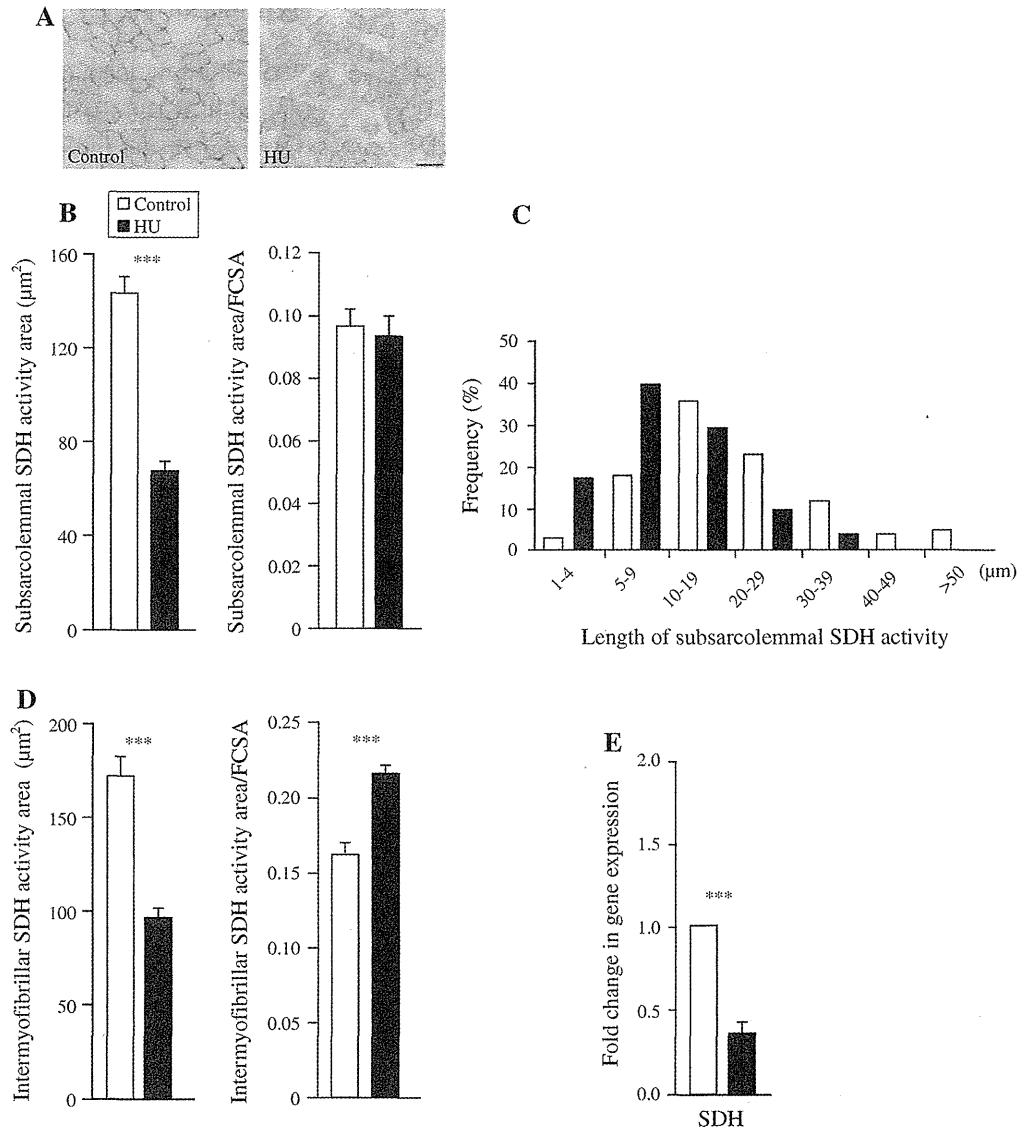

Fig. 4 Regional differences in the mitochondrial adaptations to HU. **a** Histochemical staining for SDH activity was performed on cryosections of the gastrocnemius muscle. *Scale bar* = 50 μm. **b** Quantification of SS SDH staining area in a myofiber was measured with the image-analysis system, calibrated to transform the number of pixels into micrometers. Subsarcolemmal SDH staining area in a myofiber was normalized for FCSA. **c** Quantification of length of SS SDH staining was measured. **d** Quantification of IMF SDH staining area in a myofiber was measured. Intermyofibrillar SDH staining area in a myofiber was normalized for FCSA. **e** Total RNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. The data are means ± SEM ($n = 6$). Statistically significant differences compared to control: $^{***}P < 0.001$

IMF mitochondria decrease by 73 and 45% after 5 weeks of hindlimb suspension [42]. We founded that SS mitochondria decreased in proportion to myofiber size but IMF mitochondria increased in proportion to myofiber size, suggesting that SS mitochondria were degraded more rapidly than IMF mitochondria. This phenomenon may be attributable to the development of autophagy. Riley et al. [8] indicated that autolytic degradation occurs during HU in light of the appearance of vacuolation and fragmentation of SS mitochondria.

The possible mechanism for regulating mitochondrial distribution in myofibers is not fully understood. However, it is hypothesized that abnormal distribution of mitochondria during microgravity may be due to disturbance of the structural integrity of mitochondria. In this regard, Nikawa et al. [43] reported that expression of A-kinase anchoring protein and cytoplasmic dynein, which are associated with the anchoring and movement of mitochondria, decreased in spaceflight but not in tail-suspension tests. Indeed, they did not observe abnormal distribution of mitochondria in a tail-suspended rat. This result is inconsistent with previous reports [8, 42] and our present findings. We hypothesized that this phenomenon may be associated with changes in mitochondrial morphology. In this study, therefore, we focused attention on mitochondrial morphogenesis-related genes, namely, mitofusin 2 and fission 1. Consistent with the result presented in a previous report [8], the SS mitochondria (SDH activity) in the HU case were smaller in size than those in the control case (Fig. 7b, c). Moreover, expression of mitofusin 2 decreased after HU. This is a reasonable result because this gene is regulated by PGC-1β and ERRα [44], which are down-regulated by HU. This finding is partially supported by the observation that repression of mitofusin 2 in muscle cells shows a fragmentation of the mitochondrial network [45]. However, we did not observe up-regulation of fission 1, suggesting that

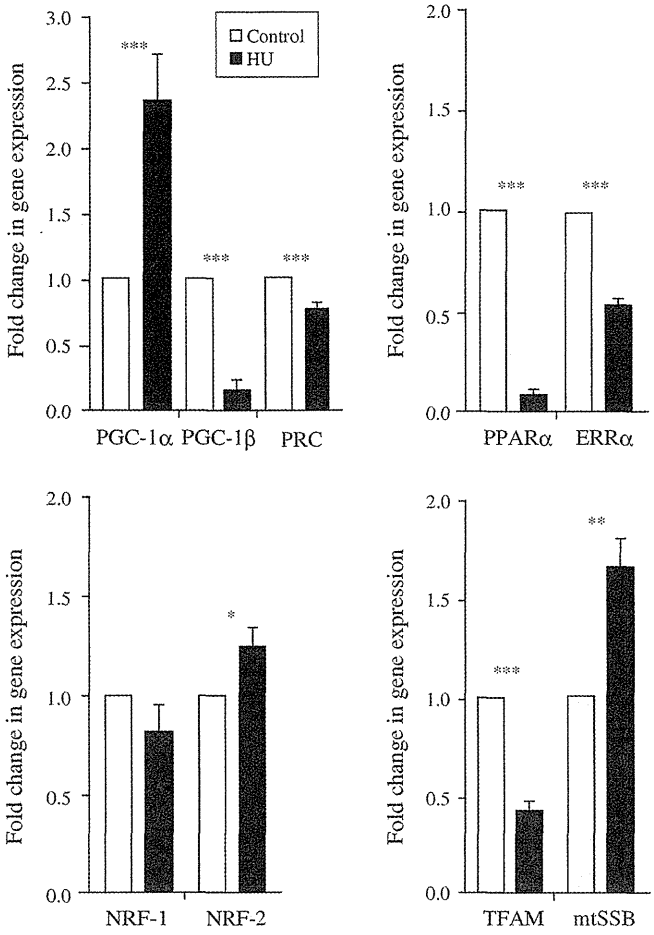

Fig. 5 Effects of HU on expression of mitochondrial biogenesis-related genes. Total RNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. The transcript levels were compared to control mice. The data are means ± SEM ($n = 6$). Statistically significant differences compared to control: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

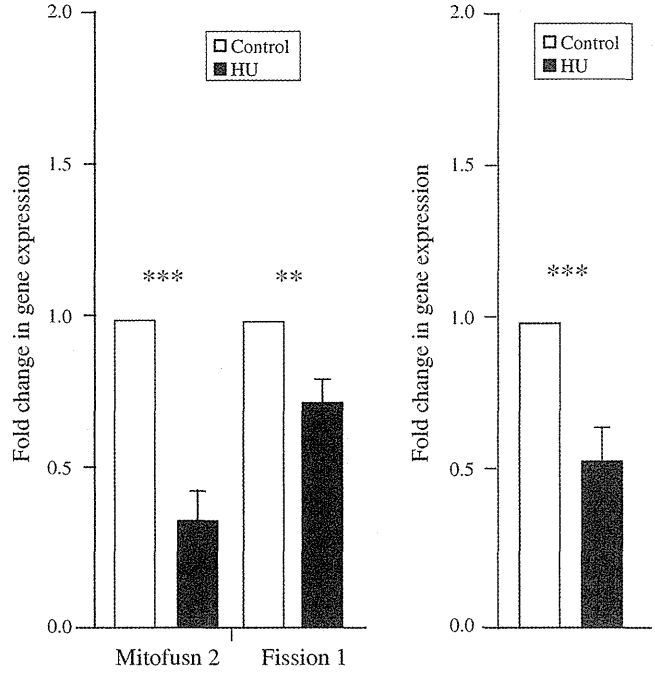

Fig. 6 Effects of HU on expression of mitochondrial morphology and mitochondrial-protein degradation-related genes. Total RNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. The transcript levels were compared to control mice. The data are means ± SEM ($n = 6$). Statistically significant differences compared to control: $^{**}P < 0.01$ and $^{***}P < 0.001$

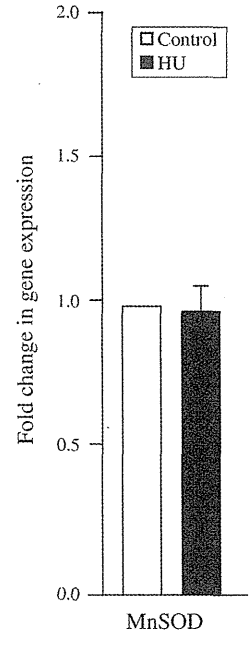

Fig. 7 Effects of HU on mRNA expression levels of mitochondrial antioxidant enzyme. Total RNA was prepared from muscle tissues and relative gene expression was determined by real-time PCR. The transcript levels were compared to control mice. The data are means ± SEM ($n = 6$)

mitochondrial fragmentation induced by HU may be caused by down-regulation of mitofusin 2 irrespective of the expression level of fission 1. However, there is direct evidence that over-expression of fission 1 induces mitochondrial fragmentation of myofibers [46]. This study therefore cannot rule out the possibility that fission 1 may contribute to fragmentation of SS mitochondria during HU. In addition, another mitochondria fission-related gene, dynamin 1-like, may play a role in mitochondrial fragmentation during HU.

Unexpectedly, we observed up-regulation of PGC-1α after HU. This finding is inconsistent with that previously reported by Mazzatti et al. [47] who showed decreased expression of PGC-1α after 24 h of HU. This seemingly contradictory finding implies that the response of PGC-1α may differ in the cases of acute and subacute HU. We attribute this discrepancy to the time course of alterations in electromyographic (EMG) activity during HU. The EMG activity of lateral gastrocnemius decreases by half immediately after HU, stays lower than a control levels for 4–5 days, and subsequently tends to recover by 7 days [48], suggesting that recovery of EMG activity may contribute to augmentation of PGC-1α expression as observed

in this study. There may be several potential intracellular signaling pathways by which PGC-1α gene expression could be regulated during HU. First, calcium signaling pathway may contribute to PGC-1α gene expression because HU promotes Ca^{2+} accumulation in myofibers [49]. Calcium/calmodulin-dependent protein kinase IV likely induces PGC-1α expression by activating cyclic-AMP-responsive-element-binding protein (CREB), which in turn binds to a conserved CRE in the PGC-1α promoter [50]. This pathway is also driven by calcineurin A through myocyte enhancer factor 2, which is a potent *trans*-activator of PGC-1α transcription in skeletal muscle [50]. Interestingly, expression levels of calcineurin mRNA and protein are elevated with HU [51]. Therefore, increased expression of PGC-1α during HU may be explained, at least in part, by activating calcium signaling. Second, HU leads to release of catecholamines [52] that can activate β-adrenergic receptors in skeletal muscle [53]. Activation of these receptors increases intracellular cAMP levels and potentially could activate CREB function on the PGC-1α promoter in skeletal muscle as it does in liver [54]. However, because levels of catecholamine were peaked at 12 h after HU, it is uncertain whether catecholamine-mediated pathway contributes to increased expression of PGC-1α as observed in this study. Third, PGC-1α is also induced via cGMP-dependent signaling resulting from elevated levels of nitric oxide (NO) [55]. It has recently been reported that NO and AMP-activated protein kinase (AMPK) act synergistically to up-regulate PGC-1α mRNA expression in vitro [56]. However, HU decreases expression levels of neuronal nitric oxide synthase protein and mRNA [57] while it increases AMPK activity [58]. Therefore, it remains uncertain as to whether the synergistic effect of NO and AMPK on PGC-1α expression during HU. It is very likely that other signaling cascades also may contribute to PGC-1α expression during HU. Further study would be needed to elucidate the possible mechanisms controlling PGC-1α expression during HU.

It is generally assumed that during HU, expression of genes involved in lipid metabolism is decreased, whereas expression of genes involved in glycolytic enzymes and glycogen synthesis is increased. This hypothesis suggests that switching between lipid usage and carbohydrate usage occurs to meet the metabolic demands of the myofibers during HU. In contrast to PGC-1β and PRC, which were down-regulated by HU, PGC-1α was up-regulated concomitant with decreased expression of its DNA binding transcription factors, PPARα and ERRα, which potently induce fatty-acid oxidation genes, MCAD and carnitine palmitoyltransferase I [59, 60]. Our findings may indicate that PGC-1α does not function harmoniously as a coactivator owing to down-regulation of PPARα and ERRα, leading to reduced expression of fatty-acid metabolism-related genes. This hypothesis may be supported by our results (Fig. 7) and previous articles reports that hindlimb suspension down-regulates gene expression of proteins involved in fatty-acid oxidation [34, 61]. Furthermore, we observed that HU decreased the activity of 3-HAD involved in the β-oxidation of fatty acids. Indeed, Grichko et al. [62] reported that fatty-acid oxidation by isolated mitochondria after 2 weeks of hindlimb suspension is on a declining trend although the difference failed to attain statistical significance. Because our result and previous study [42] show that HU decreases the absolute volumes of mitochondria, we suppose that it may also reduce fatty-acid oxidation of myofibers.

It is well established that TFAM plays a key role in mammalian mtDNA transcription/replication [16], whereas mtSSB contributes to its replication, repair, and recombination [63]. There was no alteration in expression of NRF-1, which is activated by PGC-1α and ERRα but expression of its downstream target gene, TFAM was down-regulated after HU. This down-regulation could contribute to mitochondrial dysfunction by reducing template availability for transcription and translation of key mitochondrial proteins. We observed up-regulation of mtSSB concomitant with moderately increased expression of NRF-2, which is shown to potently activate human mtSSB gene expression [64]. It was shown that mtDNA may accumulate more oxidative DNA damage relative to nuclear DNA [65]. mtSSB up-regulation may therefore be a compensatory response to oxidative stress-induced mtDNA damage during HU.

Lon protease level in skeletal muscle decreases with oxidative stress [66]. Because HU increases oxidative stress [67, 68], our finding may be explained by the oxidative stress induced by HU. This study is, to the best of our knowledge, the first to deal with the potential regulation of Lon protease after HU. It is also possible that down-regulation of Lon protease may be responsible for the accumulation of oxidatively damaged proteins within mitochondria, resulting in impairment of mitochondrial function. For example, aconitase, Kreb's cycle enzyme, is one of many mitochondrial matrix proteins that are preferentially degraded by Lon protease after oxidative modification [69]. In addition to the role of protein degradation, Lon protease can also act as a chaperone independently of its proteolytic activity [19], participate in the regulation of mitochondrial gene expression and genome integrity [70, 71], and regulate apoptotic cell death [19], suggesting that Lon protease may play some role in mitochondrial adaptations to HU. Accordingly, further studies are required to elucidate the role of Lon protease in mitochondrial adaptations to HU.

Besides being the primary site of fuel metabolism and ATP production, mitochondria are also a primary source of ROS. Mitochondria also signal, via ROS and Ca^{2+}, and are

critical regulators of cell death pathways [1]. We measured the expression of MnSOD, a primary mitochondrial antioxidant enzyme involved in quenching ROS concentrations. We hypothesized that if mitochondria of HU mice had a greater antioxidant enzyme activity, this could serve to offset elevated ROS production and reduce ROS-induced damage within mitochondria. However, we found no difference in the expression of MnSOD. This observation is in agreement with previous study reported by Andrianjafiniony et al. [72], who found no change in MnSOD activity after 14 days of HU. This does not preclude the possibility that other antioxidant enzymes may be expressed during HU. Indeed, the activities of copper–zinc superoxide dismutase and catalase are increased in hindlimb-unloaded rat [72]. The lack of adaptation of MnSOD activity to increased oxidative stress by HU suggest that a balance between respiration and ROS generation in mitochondria may be lost, leading to mitochondria-mediated apoptosis. Apoptosis can be evoked by ROS-induced mitochondrial release of the proapoptotic proteins. IMF mitochondria release a great amount of cytochrome c and apoptosis-inducing factor in response to oxidative stress compared with SS mitochondria [73], suggesting that not all mitochondria within a myofiber behave similarly. Given that IMF mitochondria were slowly decreased than SS mitochondria during HU, IMF mitochondria may play a major role in initiating apoptosis.

In conclusion, HU distorts gene expression concerning mitochondrial biogenesis, mitochondrial morphogenesis, and mitochondrial-protein degradation, resulting in changes in the abundance, morphology, and functional properties of mitochondria. Unexpectedly, PGC-1α expression is up-regulated after HU. It is unlikely that the augmentation would contribute to mitochondrial biogenesis, because several genes controlled by PGC-1α are down-regulated, suggesting that coordinated expression of PGC-1 family coactivators and DNA binding transcription factors is required for maintaining mitochondrial biogenesis in skeletal muscle. Furthermore, down-regulation of mitochondrial morphology-related genes associated with HU may be also involved in alterations in intracellular mitochondrial distribution. Our findings provide insight into the mitochondrial adaptation in skeletal muscle to HU. Interestingly, Romanello et al. [46] suggest that mitochondrial remodeling contributes to muscle atrophy. Mitochondrial adaptations may therefore have to be considered as an important event when interpreting the results of HU experiments.

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