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Knockdown of Hypoxia-Inducible Factor-1α by siRNA Inhibits C2C12 Myoblast Differentiation

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Abstract We analyzed the role of Hypoxia-inducible factor (HIF)-1α in myoblast differentiation by examining the expression and regulation of HIF-1α in proliferating and differentiating C2C12 myoblast, and by knocking down HIF-1α of C2C12 myoblasts with small interfering RNA (siRNA), given that HIF-1α has been shown to be involved in differentiative process in non-muscle tissues. Although HIF-1α mRNA was constantly expressed in C2C12 myoblasts both under growth and differentiating phase, HIF-1α protein was hardly detectable in the growth phase but became detectable only during myogenic differentiation even under normoxia. During early stage of C2C12 myogenesis, HIF-1α accumulated in the nuclei of myogenin-positive myoblasts. The inhibition of proteasome in the growth phase led to HIF-1α protein accumulation, whereas in the differentiation phase the inhibition of Hsp90, which stabilizes HIF-1α, suppressed HIF-1α accumulation. Therefore, we suggest that the level of HIF-1α protein expression is regulated by a proteasome-and chaperon-dependent pathway in C2C12 myoblast. Knockdown of HIF-1α effectively blocked myotube formation and myosin heavy chain (MHC) expression. Finally, HIF-1α expression in vivo was confirmed in the regenerative muscle tissue of mice after eccentric exercise. We conclude that HIF-1α is required for C2C12 myogenesis in vitro, and suggest that HIF-1α may have an essential role in regenerative muscle tissue in vivo. J. Cell. Biochem. 98: 642–649, 2006.

Key words: HIF-1α; HSP90; myogenesis

Muscle development is a multi-step process that begins with the determination of myogenic precursors from mesodermal stem cells and concludes with the differentiation of committed undifferentiated myoblasts [Charge and Rudnicki, 2004]. Skeletal muscle differentiation is characterized by the induction of muscle-specific gene expression in myoblasts and cell fusion into multinucleated myotubes. The muscle regulatory transcription factors (MRFs) MyoD, myogenin, MRF4, and Myf5 were initially identified as master regulators of myogenic differentiation [Braun et al., 1989; Rhodes and Konieczny, 1989; Wright et al., 1989; Rudnicki et al., 1993]. In addition to the above myogenic transcriptional factors, several others such as Pax7 [Seale et al., 2000], AP-1 [Bengal et al., 1992], NF-κB [Guttridge et al., 2000], and FOXO1a [Bois and Grosveld, 2003] were reported to be associated with myogenesis.

Adult mammalian skeletal muscle has an exceptional ability to complete a rapid and extensive regeneration in response to severe damage such as excessive mechanical stimuli, ischemia, or toxicological tissue destruction [Charge and Rudnicki, 2004]. Physical exercise is one of the factors that induce muscle damage and regeneration. While muscle contraction is largely dependent on oxidative phosphorylation utilizing oxygen, the partial oxygen tension in skeletal muscle tissue during exercise has been shown to be very low [Hoppeler et al., 2003]. Low-intensity resistance training with moderate vascular occlusion was shown to effectively increase the cross-sectional muscle area in comparison with training without vascular occlu-

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sion, suggesting a favorable effect of lower oxygen tension [Takarada et al., 2000]. Thus, local hypoxia in muscle may serve as a favorable stimulus for muscle development and regeneration.

Hypoxia-inducible factor (HIF)-1α, composed of basic-helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) proteins, is a transcription factor that senses low oxygen availability and enhances the activation of the hypoxia-inducible genes involved in energy/iron metabolism, angiogenesis, and erythropoiesis [Semenza, 2003; Lee et al., 2004]. Several investigators have demonstrated that HIF-1α activity is also crucial for normal tissue development and functional organ assembly through proliferation, survival, and/or differentiation in murine non-muscular tissues [Iyer et al., 1998; Caniggia et al., 2000; Yun et al., 2002; Seagroves et al., 2003; Tomita et al., 2003]. For instance, HIF-1α plays a critical role in hypoxia-mediated trophoblast differentiation through TGF-β3 inhibition [Caniggia et al., 2000]. HIF-1α was also shown to be an essential regulator for the secretory differentiation and activation of murine mammary epithelium [Seagroves et al., 2003]. HIF-1α not only favors differentiation but could suppress differentiation as such in the hypoxia-mediated inhibition of adipogenesis through the induction of DEC1/Stra13 expression [Yun et al., 2002]. Thus, HIF-1α function appears to be cell- or organ-type-specific with different consequences depending on the cell or organ type.

Although lower oxygen tension favors skeletal muscle regeneration or development and HIF-1α accumulation is detected in the skeletal muscle tissue, the role of HIF-1α in skeletal muscle has not yet been established. To understand how myogenesis is coordinated with HIF-1α-mediated cellular adaptation, we examined the expression of HIF-1α during myogenesis in C2C12 myoblasts and observed the effect of the transient knockdown of HIF-1α on myogenic differentiation by means of siRNA. We show that HIF-1α may be one of the crucial transcriptional factors in the regulation of C2C12 myogenesis.

METHODS

Antibody and Reagent

Rabbit anti-HIF-1α polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-Hsp90 mAb was obtained from Stressgen (Victoria, Canada). Mouse anti-MHC (MF20) mAb, mouse anti-myogenin (F5D) mAb, and mouse anti-β-tubulin (E7) mAb were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa, Development of Biological Science (Iowa City, IA). Topro-3, Alexa Fluor488-conjugated goat anti-mouse IgG, Alexa Fluor568-conjugated goat anti-rabbit IgG, Alexa Fluor488-conjugated goat anti-rat IgG, and HRP-conjugated goat anti-mouse IgG were obtained from Invitrogen Corp. (Carlsbad, CA). HRP-conjugated goat anti-rabbit IgG was obtained from Cell Signaling Technology (Beverly, MA). Proteasome inhibitor MG132 was obtained from Calbiochem (Darmstadt, Germany) and dissolved in DMSO. Hsp90 inhibitor geldanamycin (GA) was purchased from BIOMOL (Plymouth Meeting, PA) and dissolved in DMSO.

Cell Culture and Induction of Myogenic Differentiation

A mouse embryonic myoblastic cell line, C2C12 (RCB0987), was provided by Riken Cell Bank (Tsukuba, Japan). It was grown in growth medium (GM; Ham's F-10 medium (Invitrogen Corp.) containing 20% fetal bovine serum and 1% penicillin/streptomycin). Myogenic differentiation was induced by serum deprivation. Cells were washed with PBS, and the culture medium was replaced with differentiation medium (DM; Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 2% horse serum and 1% penicillin/streptomycin) at 37°C in a humidified incubator under 5% CO_2. All of the experiments were carried out from day 0 to day 3 after the induction of differentiation, and cells were examined daily for biochemical and morphological signs of myogenesis. Cell viability was examined by trypan blue dye exclusion method. We examined in a preliminary experiment that cell viability even after MG132 and geldanamycin treatment at all dose used in the experiment was more than 95%.

RNA Interference

The transfection of siRNA to C2C12 cells was performed twice in six-well plates. The first transfection with HIF-1α siRNA or control siRNA was performed when the cells were 50–60% confluent in GM. At 48 h post-transfection,

when the cells were 80–90% confluent, the procedure was repeated. Both HIF-1α and a control siRNA duplex called stealth siRNA were obtained from Invitrogen Corp., and the duplexes were resuspended to 6 μM. For transfections, siRNA duplexes were incubated with Lipofectamine 2000 (Invitrogen Corp.), according to the manufacturer's instructions. Differentiation was induced after the second transfection by serum deprivation as described above. The mouse HIF-1α siRNA sequence used was 5′-AAGCAUUUCUCUCAUUUCCUCAUGG-3′ and the control siRNA sequence used was 5′-AAGACCUUUAUCUCUUACUCCUUGG-3′.

Western Blot Analysis

C2C12 cells were washed with PBS and lysed with 1 ml of a lysis buffer (40 mM Tris (pH 7.5), 300 mM KCl, 1% Triton X-100, 2 mM EDTA, and protease inhibitor cocktail (1:50; Sigma) for 40 min on ice. The extracts were cleared by centrifugation at 15,000*g* for 30 min. Equal amounts of protein in the supernatant (10–30 μg protein/lane), estimated by the Lowry method using a protein assay, were electrophoresed on a 10% SDS gel and sequentially electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% non-fat milk in PBS containing 0.1% Tween 20 for 1 h at room temperature, membranes were incubated with either anti-MHC mAb (1:200), anti-myogenin mAb (1:400), anti-β-tubulin mAb (1:200), or anti-HIF-1α polyclonal antibody (1:300) in TBS (pH 8.0) containing 0.05% Tween 20 (TBST) at 4°C overnight and washed three times for 5 min with PBS. Then the membranes were incubated with HRP-conjugated secondary anti-mouse IgG (1:1,000) or anti-rabbit IgG (1:2,000) in TBST for 1 h at room temperature and washed three times for 5 min with PBS, then visualized by chemiluminescence by using an ECL immunoblotting kit (Cell Signaling Technology) with a digital luminescent image analyzer LAS-1000 (Fujifilm, Tokyo, Japan). Band intensity was assessed by using Scion Image (Frederick, MD).

RNA Preparation and Semi-Quantitative Reverse Transcriptase (RT)- PCR

Total RNA was extracted from C2C12 cells by using ISOGEN reagent (Nippongene, Tokyo, Japan), according to the manufacturer's specifications, and stored frozen at −80°C until needed. One microgram of extracted total RNA in a 20 μl volume of each sample was reverse transcripted to obtain cDNA using a RT Kit Rever Tra Dash (TOYOBO, Osaka, Japan). One microliter cDNA template in a total volume of 25 μl containing Taq DNA polymerase (Invitrogen Corp.) was amplified. PCR amplification conditions were 94°C for 30 s, 55°C for 1 min, 72°C for 1 min for 22 cycles, with the last cycle including an extension for 10 min at 72°C. Primers for the detection of RT products were derived from different exons to distinguish RT-PCR products from genomic DNA contaminations. The primer sequences used were mouse HIF-1α 5′-TGCTCATCAGTTGCCACTT-3′ (forward) and 5′-TGGGCCATTTCTGTGTGTA-3′ (reverse); mouse GAPDH, as an internal control, 5′-TTGTGATGGGTGTGAACCAC-3′ (forward) and 5′-ACACATTGGGGGTAGGAACA-3′ (reverse). The PCR products were separated on 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized with a UV transilluminator.

Immunocytochemistry

Immunocytofluorescence staining was carried out in eight-well chamber slides (Nunc, Inc., Naperville, IL). C2C12 cells were fixed in PBS containing 4% paraformaldehyde at room temperature for 20 min, and were blocked and penetrated in PBS containing 5% bovine serum albumin (BSA) and 0.3% Triton X-100 for 20 min at room temperature, then incubated overnight with either anti-Hsp90 mAb (1:300), anti-myogenin mAb (1:300), or anti-HIF-1α polyclonal antibody (1:100) in TBS (pH 8.0) containing 0.01% Triton X-100 at 4°C overnight. Washes were done with PBS. Then, the cells were incubated with secondary antibodies coupled to Topro-3 as a nuclear staining control in TBS (pH 8.0) containing 0.03% Triton X-100 for 1 h at room temperature. The reaction was examined by confocal microscopy (Nicon, Tokyo, Japan).

Immunohistochemistry

Frozen gastrocnemius muscle cross-sections were fixed in PBS containing 4% paraformaldehyde at room temperature for 20 min, and blocked for 1 h at room temperature in PBS containing the blocking reagent of the M.O.M kit (Vector Laboratories, Burlingame, CA). Sections were then incubated with primary antibodies, anti-HIF-1α polyclonal antibody (1:100) and

anti-myogenin mAb (1:300) in TBS (pH 8.0) containing 0.03% Triton X-100 and the dilution reagent of the M.O.M kit at 4°C overnight. Washes were done with PBS. Then, sections were incubated with the secondary antibodies, Alexa Fluor488-conjugated goat anti-mouse IgG (1:400) and Alexa Fluor568-conjugated goat anti-rabbit IgG (1:400) coupled to Topro-3 in TBS (pH 8.0) containing 0.03% Triton X-100 and the dilution reagent of the M.O.M kit for 1 h at room temperature. The reaction was examined using confocal microscopy (Nicon).

Animals and Muscle Damage Protocol

Adult male mice (C57BL/6N, 8-week old, n = 5) were housed in a cage at 20–23°C under a 12: 12-h dark-light cycle. This study was performed according to the Guidelines and Regulations for Laboratory Animal Care of Tohoku University Graduate School of Medicine.

The mice were anesthetized by an intraperitoneal injection of pentobarbital sodium at 50 mg/kg body mass. The fur of the right hind limb was removed. The right hind limb was fixed at the knee, and the right foot was strapped with adhesive tape to a metal plate attached to a servomotor (RU72, NEC, Japan). Contractions of the triceps sural muscle of the right lower leg were induced using an electrical stimulator (SEN3301, Nihon Kohden, Japan) with 0.5 ms pulses at 150 Hz and 15 V for 0.6 s, via surface electrodes attached on the medial and lateral sides of the limb. During the 0.6 s activation period, the ankle was forced to dorsiflex by 90° from the position of maximum plantar flexion, which resulted in an eccentric contraction of the triceps sural muscle. The action was repeated every 4 s, for five sets of 60 repetitions, with a 5 min rest between the sets. The contralateral limb was used as a non-exercised control. After eccentric contraction, the gastrocnemius muscle was rapidly isolated, embedded in O.C.T. compound, and frozen in liquid nitrogen, then stored at −80°C until measurement. Serial 8-μm thick frozen gastrocnemius muscles were cross-sectioned with a microtome cryostat at day 5 after eccentric contractions.

RESULTS

HIF-1α Expression During C2C12 Differentiation

To investigate HIF-1α induction during muscle differentiation in vitro, we used C2C12 mouse embryonic myoblasts. In GM, C2C12 myoblasts proliferated until they became confluent. Full differentiation into multinucleated myotubes was obtained by culturing 80–90% confluent C2C12 cells in DM. As shown in Figure 1A, we confirmed that myotube differentiation markers such as myosin heavy chain (MHC) and myogenin became detectable on day 1 and day 3 after the cells were placed in DM. The level of HIF-1α protein also increased from day 1 to day 3 under the differentiating culture

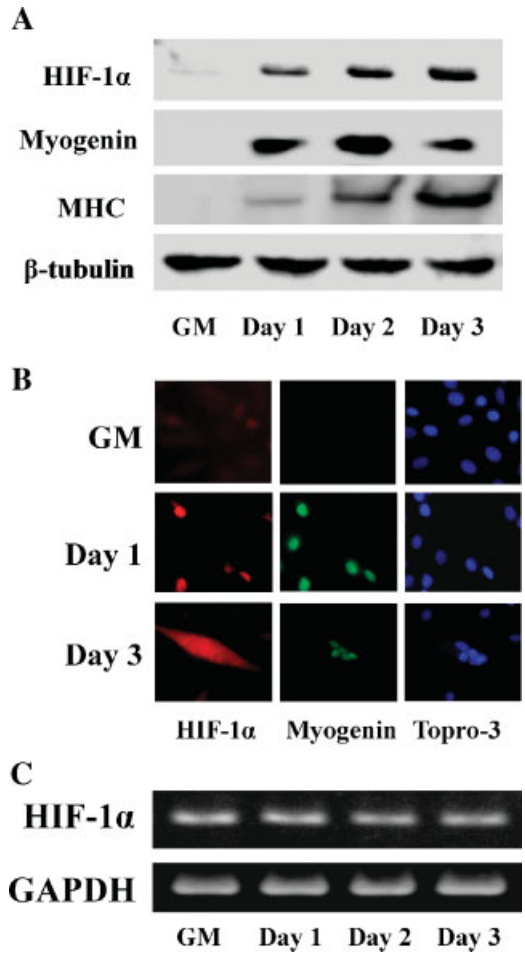

Fig. 1. HIF-1α expression during C2C12 differentiation. **A**: Western blot analysis of HIF-1α protein during myogenesis. HIF-1α began to increase on day 1 after the induction of myogenic differentiation. MHC and myogenin were used as the markers of myogenesis. **B**: Confocal microscopic analysis for the distribution of HIF-1α protein during myogenesis. HIF-1α accumulation was observed in the nucleus stained with Topro-3 of myogenin-positive myoblasts on day 1 of differentiation. At mature stage day 3, HIF-1α was expressed both in the nucleus and in the cytoplasm. **C**: RT-PCR of HIF-1α mRNA during myogenesis. The relative amount of HIF-1α mRNA was constant regardless of the culture conditions. A representative data of at least three independent experiments with similar results is shown.

condition. Next, we examined the distribution of HIF-1α during C2C12 myogenesis by using confocal microscopic analysis. HIF-1α accumulated in the nucleus of myogenin-positive myoblasts on day 1 of differentiation. At a more maturated stage on day 3, HIF-1α was expressed both in the nucleus and in the cytoplasm (Fig. 1B). However, the amount of HIF-1α mRNA was constant regardless of the culture conditions (Fig. 1C).

Proteasome Inhibitor MG132 Causes Accumulation of HIF-1α Under GM

In order to examine whether or not HIF-1α is degraded by proteasome, we examined the effect of MG132, a specific inhibitor of the ubiquitin proteasome complex, on HIF-1α degradation. Treatment with 1 μM or 10 μM MG132 for 4 h under the growth condition resulted in HIF-1α accumulation as detected by immunoblot analysis (Fig. 2).

Hsp90 Activity Is Required for HIF-1α Induction During Myogenesis

We examined whether Hsp90 protects HIF-1α from proteasome degradation during myogenic differentiation of C2C12 cells. Figure 3A shows the confocal microscopic analysis of Hsp90 during C2C12 differentiation. Hsp90 was upregulated from day 1 to day 3 of differentiation, and co-localized with HIF-1α. Moreover, pharmacological inhibition of Hsp90

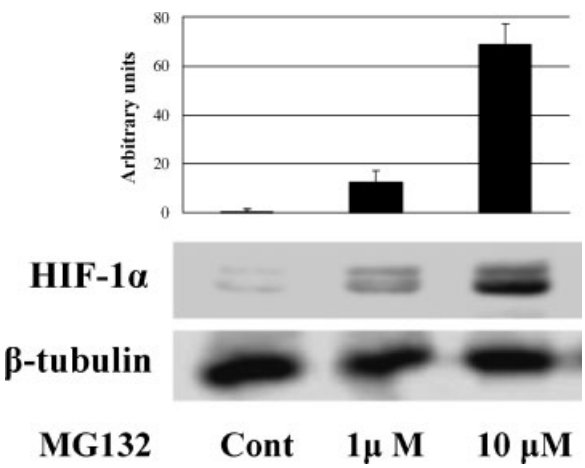

Fig. 2. Proteasome inhibitor MG132 causes accumulation of HIF-1α under GM. The effect of MG132, a specific inhibitor of the ubiquitin proteasome, on HIF-1α degradation. Treatment with 1 μM or 10 μM MG132 for 4 h under the GM condition resulted in HIF-1α protein accumulation as detected by Western blot analysis. The data were repeated at least three times, and SD is shown as bars.

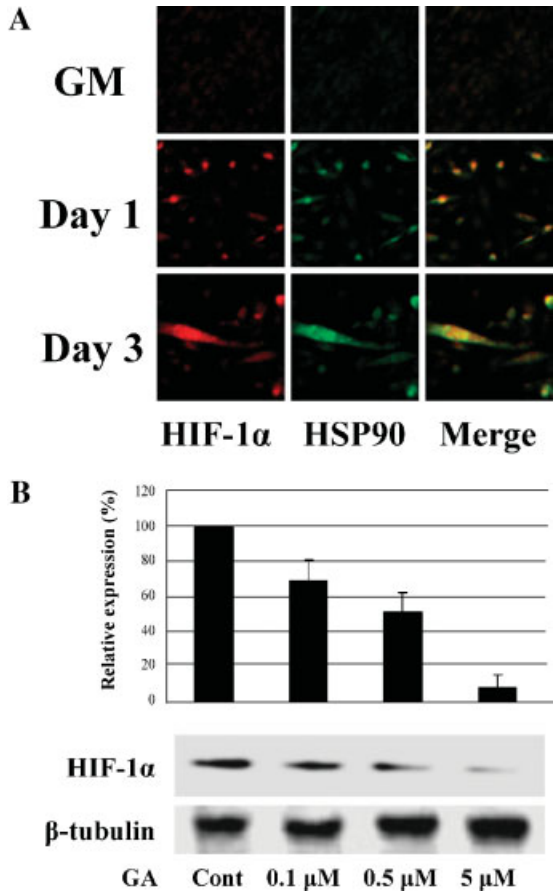

Fig. 3. Hsp90 activity is required for HIF-1α induction during myogenesis. **A**: Confocal microscopic analysis for the distribution of Hsp90 during myogenesis. Hsp90 was upregulated from day 1 to day 3 of differentiation, and co-localized with HIF-1α. **B**: The effect of pharmacological inhibition of Hsp90 by means of GA for 4 h on HIF-1α expression on day 1 of differentiation. The treatment with GA markedly reduced HIF-1α expression in a dose-dependent manner. The data were repeated at least three times, and SD is shown as bars.

by geldanamycin markedly reduced HIF-1α accumulation in a dose-dependent manner on day 1 of differentiation (Fig. 3B).

HIF-1α Knockdown Inhibits C2C12 Differentiation

To examine the role of HIF-1α in C2C12 differentiation, an RNA interference technique was employed to specifically knockdown HIF-1α mRNA. Two siRNA duplexes were prepared, HIF-1α siRNA and control siRNA. The expression of HIF-1α protein was reduced up to 30% from day 1 to day 3 (Fig. 4A). The observed HIF-1α knockdown led to a significant reduction in MHC expression. In addition, multinucleated myotubes were clearly visible in control cells

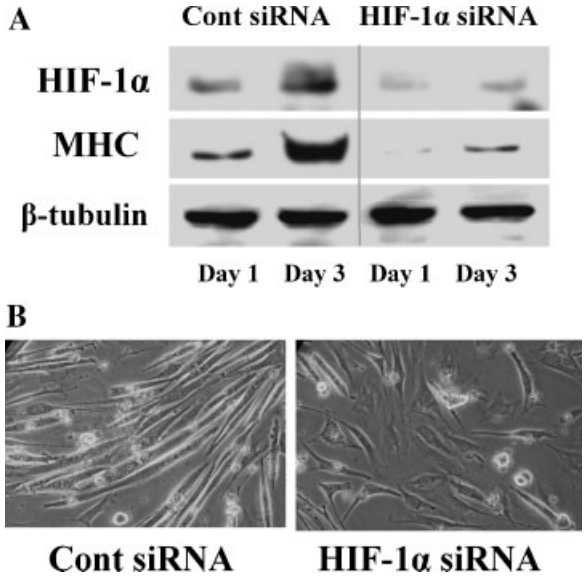

Fig. 4. HIF-1α knockdown inhibits C2C12 differentiation. **A**: Western blot analysis of the effect of HIF-1α siRNA on myogenic differentiation. HIF-1α knockdown led to a significant reduction in MHC expression. **B**: Morphological analysis of the effect of HIF-1α siRNA on myogenic differentiation. Multinucleated myotubes were clearly visible in the cells treated with control siRNA under the light microscope but were almost undetectable in the cells treated with HIF-1α siRNA. A representative data of at least three independent experiments with similar results are shown.

under the light microscope, but were almost undetectable in the cells treated with HIF-1α siRNA (Fig. 4B). Taken together, these data indicate that HIF-1α supports myoblast differentiation.

Induction of HIF-1α Expression in Regenerating Muscle In Vivo

To examine whether HIF-1α is involved in muscle regeneration in vivo, we stained HIF-1α in damaged muscle tissue after eccentric muscle contraction (Fig. 5). At day 5 after the induction of muscle damage, HIF-1α was found to co-localize with myogenin in the damaged tissue. In the control muscle, there was little or no immunoreactivity for HIF-1α.

DISCUSSION

We observed varying levels of HIF-1α protein, which decreased in the growth phase and increased in the differentiation phase, in contrast to the continuous expression of HIF-1α mRNA regardless of the culture conditions in C2C12 myoblasts. In an earlier study, it was reported that MG132, a specific inhibitor of proteasome, enhanced HIF-1α transcriptional activity in L6 myoblasts [Zelzer et al., 1998]. We also confirmed that MG132 treatment of C2C12 myoblasts resulted in a significant accumulation of HIF-1α protein even under the growth condition. This result shows that the HIF-1α protein level is kept low during the growth phase due to continuous degradation by proteasome.

Under normoxia, HIF-1α protein is known to be ubiquitinated by E3 ubiquitin ligase VHL in various cell types [Huang et al., 1998; Iwai et al., 1999; Lisztwan et al., 1999; Maxwell et al., 1999; Ohh et al., 2000]. Under hypoxia, VHL is unable

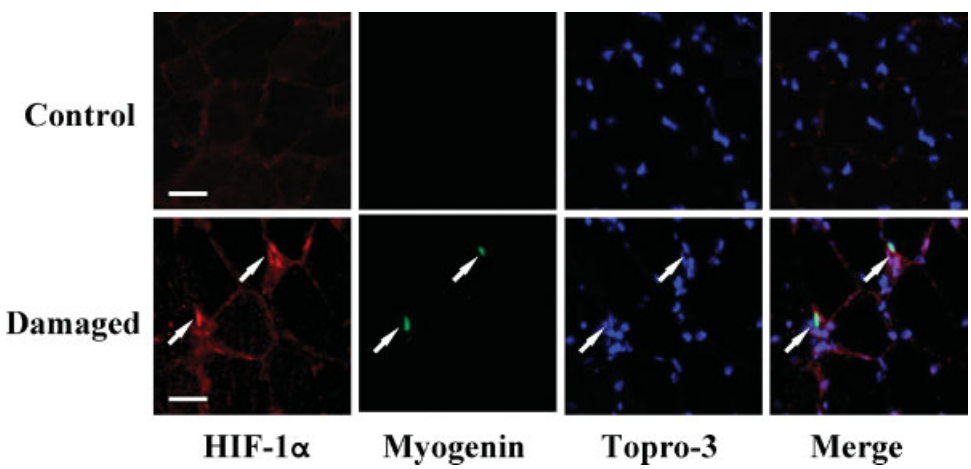

Fig. 5. Induction of HIF-1α expression in regenerating muscle in vivo. Confocal microscopic analysis of damaged muscle tissue on day 5 after eccentric muscle contractions. At day 5 after the induction of muscle damage, HIF-1α co-localized with myogenin in the damaged tissue (arrow). Bar = 60 μm

to associate with and ubiquitinate HIF-1α. HIF-1α then escapes from degradation, translocates to the nucleus, and activates the transcription of target genes. In this study, however, we demonstrated an upregulated expression of HIF-1α protein during C2C12 muscle differentiation, even under normoxia. This result suggests that HIF-1α is stabilized independently of oxygen tension during C2C12 myogenesis.

One of the chaperone proteins, Hsp90 is known to protect newly synthesized HIF-1α from oxygen-independent and VHL-independent degradation by means of an unidentified proteasome pathway without altering the steady state of HIF-1α mRNA in non-muscular cells [Mabjeesh et al., 2002; Zhou et al., 2004]. Hsp90 was shown to physically interact with HIF-1 in vitro [Minet et al., 1999; Zhou et al., 2004]. Therefore, to determine whether Hsp90 is involved in the stabilization of HIF-1α during myogenesis, we tried to inactivate Hsp90 pharmacologically using geldanamycin. We could clearly demonstrate dose-dependent inhibition of myogenesis-induced HIF-1α accumulation, which suggested that the stabilization of HIF-1α during myogenesis is dependent on Hsp90 function. The increase in Hsp90 co-localization with HIF-1α during C2C12 myogenesis, as demonstrated by confocal microscopic analysis, also supports the stabilizing effect of Hsp90 on HIF-1α. The upregulation of Hsp90 in the regeneration of the muscle fibers of Duchenne muscular dystrophy patients in vivo [Bornman et al., 1996] and its regulatory role in the myogenesis of zebrafish [Lele et al., 1999] confirms the essential role of Hsp90 in myogenesis. Thus, we suggest that Hsp90-mediated stabilization of HIF-1α is required for the process of C2C12 myogenesis.

We then tested the effect of transient siRNA knockdown of HIF-1α to determine whether HIF-1α is important in C2C12 myogenesis. HIF-1α knockdown resulted in a remarkable inhibition of C2C12 muscle differentiation both morphologically and biochemically, suggesting that HIF-1α may have an important role in myogenesis in vitro.It was reported that complete deficiency of HIF-1α resulted in developmental abnormalities such as fewer than normal somites and lethality by day 11 of HIF-$1\alpha^{-/-}$ embryos [Iyer et al., 1998]. Therefore, HIF-1α is not only important for the postnatal regeneration of muscle, but also for muscle development during early embryogenesis.

In contrast with our observations, Mason et al. [2004] have reported that skeletal-muscle-specific deletion of HIF-1α in mice did not affect the normal development of skeletal muscle [Mason et al., 2004]. However, they used muscle type creatine kinase (MCK)-Cre/loxP system for the deletion of skeletal muscle HIF-1α. Since the expression of MCK is observed in the latter stage of myogenesis [Jiang et al., 1998], the deletion of HIF-1α in skeletal muscle should occur when MCK is induced in the mature stage of muscle differentiation. Therefore, we assume that HIF-1α was not deleted in the satellite cells at an earlier stage of muscle differentiation. The fact that MCK-Cre/loxP deletion of HIF-1α did not affect normal muscle differentiation suggests that HIF-1α is required in a limited period at an earlier stage of myogenesis.

More recently, Yun et al. reported that constitutive expression of active HIF-1α in mutant C2C12 cells could not accelerate normal myogenesis [Yun et al., 2005]. Excessive HIF-1α does not seem to be necessary for myogenic differentiation, whereas a moderate amount of HIF-1α is required to induce myogenesis at certain stages of muscle differentiation.

We also demonstrated that HIF-1α expression is upregulated in regenerating fiber, whose regeneration is induced by eccentric contraction in vivo. This observation implies that HIF-1α may play an important role in skeletal myogenesis in vivo as well as in vitro.

In conclusion, we demonstrated for the first time that the expression of HIF-1α increases after myogenic induction, and that HIF-1α may be one of the crucial transcriptional factors in the regulation of C2C12 myogenesis.

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