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Hypoxia induces adipogenic differentitation of myoblastic cell lines

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ABSTRACT

Muscle atrophy usually accompanies fat accumulation in the muscle. In such atrophic conditions as back muscles of kyphotic spine and the rotator cuff muscles with torn tendons, blood flow might be diminished. It is known that hypoxia causes trans-differentiation of mesenchymal stem cells derived from bone marrow into adipocytes. However, it has not been elucidated yet if hypoxia turned myoblasts into adipocytes. We investigated adipogenesis in C2C12 and G8 murine myogenic cell line treated by hypoxia. Cells were also treated with the cocktail of insulin, dexamethasone and IBMX (MDI), which has been known to inhibit Wnt signaling and promote adipogenesis. Adipogenic differentiation was seen in both hypoxia and MDI. Adipogenic marker gene expression was assessed in C2C12. CCAAT/enhancer-binding protein (*C/EBP*) β, α and peroxisome proliferator activating receptor (*PPAR*) γ were increased by both hypoxia and MDI. The expression profile of *Wnt10b* was different between hypoxia and MDI. The mechanism for adipogenesis of myoblasts in hypoxia might be regulated by different mechanism than the modification of Wnt signaling.

1. Introduction

Fatty degeneration was often seen in the back muscles of kyphotic spine [1], rotator cuff muscles with torn tendons [2] and a variety of neuromuscular diseases including Duchenne muscular dystrophy [3], mitochondrial myopathy [4], nemaline myopathy [5], and amyotrophic lateral sclerosis [6]. In the pathogenesis of fatty degeneration, it has been reported that stem cells in the muscular tissue serve as a source of adipocytes. The pluripotent stem cells, which can differentiate into adipocytes, are known to reside in the muscle tissue [7,8].

The differentiation of skeletal muscle cells is controlled by myogenic regulatory factors, such as myogenin and MyoD [9]. On the other hand, differentiation of adipocytes is known to be controlled by peroxisome proliferator activating receptor (PPAR) γ and CCAAT/enhancer-binding protein (C/EBP) families of transcription factors [10]. Forced expression of PPARγ lead to differentiation of myoblasts into adipocytes [11]. However, the molecular mechanism to determine the expression of these transcription factors has not yet been fully clarified.

Wnt signaling has been known to control adipogenic differentiation. Suppression of Wnt signaling regulated PPARγ and C/EBPα, and induced adipogenic differentiation of muscle progenitors [12]. Activation of the canonical Wnt/β-catenin pathway inhibited differentiation and apoptosis of pre-adipocytes through PPARγ and C/EBPα [12].

Mesenchymal stem cells derived from bone marrow have been reported to differentiate into the adipocyte-like cells in hypoxic conditions (1% oxygen) [13]. However, it has not been fully elucidated yet whether myoblasts trans-differentiated under hypoxic conditions into adipocytes or not.

In the current study, we investigated the adipogenesis under hypoxia in C2C12 and G8 myogenic cell line and assessed adipogenic marker gene expression in C2C12, which were then compared to previously known adipogenetic induction medium.

2. Materials and methods

2.1. Cell culture

A murine myogenic cell line, C2C12 was obtained from RIKEN cell bank (Tsukuba, Japan) and G8 was from American Type Culture Collection (Rockville, MD, USA). The C2C12 cells were maintained in growth medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 50 U/ml penicillin, and 50 mg/ml streptomycin (Invitrogen)] in 5% CO_2 chamber at 37 °C. The G8 cells were maintained in growth medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (Invitrogen), 50 U/ml penicillin and 50 mg/ml streptomycin (Invitrogen)]. The cells were seeded on six-well

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plates at a concentration of 3×10^5 cells per well. After cells had reached confluence, cells were treated with hypoxia or adipogenic induction medium as mentioned below. Medium was changed every other day. A CO_2 multi gas incubator (ASTEC, Japan) filled with nitrogen gas was used to obtain the hypoxic condition (94% N_2, 5% CO_2, and 1% O_2). The culture was proceeded up to 7 days in hypoxia. For adipogenic differentiation, C2C12 and G8 cells were treated in an adipogenic induction medium [MDI: 0.5 mM isobutylmethylxanthine (IBMX), 1 μM dexamethasone and 10 μg/ml insulin in 4.5 g/L glucose DMEM supplemented with 10% FBS] [14]. After 72 h, the medium was changed to adipogenic maintenance medium [10 μg/ml insulin in 4.5 g/ml glucose DMEM supplemented with 10% FBS] [15,16].

2.2. Oil red-O staining and quantification

The cells were fixed with 10% neutral buffered formalin, washed with distilled water and replaced by 60% isopropanol. The cells were stained with Oil red-O solution [Oil red-O (Sigma, MO) in 60% isopropanol] for 30 min. The cultures were then washed thoroughly with distilled water. The extent of Oil red-O staining were quantified according to a previously published method [17]. Oil red-O stain was extracted in 100% isopropyl alcohol and its absorbance was measured by a spectrophotometer (Infinite M200, Tecan, Switzerland) at a wavelength of 510 nm.

2.3. Immunocytochemistry

Cultured C2C12 cells were fixed in −20° C ethanol for 10 min, and penetrated in phosphate buffered saline (PBS) containing 0.1% Tween 20 (ICN Biomedicals, CA) for 15 min at room temperature. Cells were incubated with a primary mouse monoclonal sarcomeric myosin-specific MF20 antibody (Developmental Studies Hybridoma Bank: DSHB, IA) in the PBS at 4° C overnight followed by incubation with AlexaFluor 555-conjugated gout anti-mouse IgG secondary antibody (Invitrogen, CA) in the PBS for 2 h at room temperature.

2.4. Quantitative RT-PCR

Total RNA from C2C12 cells was extracted with RNeasy Mini kit (QIAGEN). First strand cDNA was synthesized using High capacity cDNA archive kit (Applied Biosystems, CA). Quantitative RT-PCR reactions were performed using ABI StepOnePlus, Power SYBR Green PCR MasterMix (Applied Biosystems) and 500 nM of each primer, routinely in duplicate. The primers were designed based on the sequences in the GenBank database. The primer pairs used for this study include β-actin: L, 5′-gccaaccgtgaaaagatgac-3′and R, 5′-gaggcatacagggacagcac-3′; HIF1: L, 5′-gcactagacaaagttcacctgaga-3′ and R, 5′-cgctatccacatcaaagcaa-3′; EPAS1: L, 5′-ggttaaggaacccaggtgct-3.′ and R, 5′-gggatttctccttcctcagc-3′; VEGF: L, 5′-gcagcttgagttaaacgaacg-3.′ and R, 5′-ggttcccgaaaccctgag-3′; Wnt10b: L, 5′-gtggatcctgcaccctgaac-3.′ and R, 5′-cctccagcatgtcgaagc-3′; C/EBPβ: L, 5′-tgatgcaatccggatcaa-3.′ and R, 5’-cacgtgtgttgcgtcagtc-3’; C/EBPα: L, 5’-ctgagacccctcggggaac-3’ and R, 5’-aaacgtcagttcacagggaag-3’; PPARγ: L, 5’-gaaagacaacggacaaatcacc-3 and R, 5′-gggggtgatatgtttgaacttg-3; Pax7: 5′-ggcacagaggaccaagctc-3 and R, 5′-gcacgccggttactgaac-3; MyoD: L, 5′-cgctacatcgaaggtctgc-3 and R, 5′-ggtgtcgtagccattct-3; abcb1a: L, 5′-ggatcacgttctccttcacc-3 and R, 5′-caggagcgaatgaactgacc-3; abcb1b: L, 5′-ggccaagccttgaaggaca-3 and R, 5′-ggcctgggtgaaggagaac-3; abcg2: L, 5′-atgagcccacgactggtttg-3 and R, 5′-gccagtaaggtgaggctgtca-3.

The fractional cycle number at which the fluorescence passes the threshold (CT values) was used for quantification by using a comparative CT method. Sample values were normalized to the threshold value for β-actin: ΔCT = CT (experiments)−CT (β-actin). The CT value of control was used as a reference. ΔΔCT = ΔCT (experiment)−ΔCT (control). The fold change in mRNA expression was calculated by following formula: $2^{-\Delta\Delta CT}$.

2.5. Western blot analysis

Nucleoplasm of cultured cells was extracted [18]. Lysates were subjected to SDS–PAGE with 8% acrylamide gel. Samples were blotted to a polyvinylidene difluoride membrane (Bio-Rad, CA). The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and incubated with HIF (Hypoxia-inducible factor) 1α antibody (ab1: abcam, MA) at 4° C over night. HRP-conjugated secondary anti-mouse IgG (1:1000; Santa Cruz Biotechnology, CA) was applied for 2 h at room temperature. Signals were visualized by chemiluminescence using an ECL plus Western Blotting Detection Reagents (GE Healthcare, UK) with a digital luminescent image analyzer LAS-1000 (Fujifilm, Japan). Band intensities were analyzed by ImageJ 1.42 software (National Institutes of Health, MD).

2.6. Statistical analysis

Data were presented as means ± SEM of independent experiments. To assess the effect of adipogenesis on temporal data, analyses of unpaired Student's *t*-test were used to determine significant differences between mRNA levels of control and hypoxia or MDI. *p* values less than 0.05 were considered as statistically significant.

3. Results

3.1. Expression of hypoxia related factors

HIF1, endothelial PAS domain protein (EPAS) 1/HIF2 and vascular endothelial growth factor (VEGF) mRNA expression profiles were analyzed by quantitative RT-PCR after 2-day treatment under hypoxia or MDI. The mRNA expression of HIF1 and EPAS1/HIF2 did not show any significant differences in hypoxia or MDI. On the other hand, a significant increase of VEGF mRNA expression was seen in hypoxia (Fig. 1A). Western blotting analysis confirmed the nuclear accumulation of HIF1α protein in hypoxia. In MDI, the nuclear HIF1α accumulation was slightly higher than control but not significant (Fig. 1B).

3.2. Expression of Wnt signaling

Wnt10b mRNA expression profiles were assessed by quantitative RT-PCR, to analyze the difference in molecular mechanisms of Wnt signaling between control and hypoxia, or control and MDI (Fig. 1 C). Wnt10b mRNA expression exhibited significant increase from 24 h in hypoxia and significant decrease from 12 h in MDI.

3.3. Adipogenic differentiation of C2C12 and G8 cells

C2C12 and G8 cells cultured in hypoxia or adipogenic induction and maintenance medium (MDI) for 7 days showed positive staining of Oil red-O. The control cells exhibited subtle staining with Oil red-O (Fig. 2 A-C. E-G). The extent of Oil red-O staining of C2C12 and G8 showed significant increase in both hypoxia and MDI (Fig. 2 D. H).

*C/EBP*β, *C/EBP*α and *PPAR*γ mRNA expression profiles were assessed by quantitative RT-PCR, to determine the difference in molecular mechanisms of adipogenesis between control and hypoxia, or MDI (Fig. 2 I). *C/EBP*β mRNA expression exhibited significant increase from 12 h in both hypoxia and MDI. A significant increase of *C/EBP*α mRNA expression was seen at 48 h in hypoxia, from 12 h in MDI. *PPAR*γ mRNA expression showed a significant increase from 36 h in both hypoxia and MDI.