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A FoxO1-dependent, but NRF2-independent induction of heme oxygenase-1 during muscle atrophy

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ABSTRACT

Skeletal muscle plays key roles in metabolic homeostasis. Loss of muscle mass, called muscle atrophy exacerbates disease-associated metabolic perturbations. In this study, we characterized the molecular functions and mechanisms underlying regulation of skeletal muscle atrophy induced by denervation. Denervation significantly increased the expression of heme oxygenase-1 (HO-1) and atrogenes in skeletal muscle. Forkhead box protein O1 (FoxO1) drastically increased in atrophied muscle and selectively stimulated HO-1 gene transcription through direct DNA binding. Lack of HO-1 substantially attenuated muscle atrophy, whereas HO-1 overexpression caused muscle damage in vitro and in vivo. Collectively, HO-1 induced by FoxO1 may cause skeletal muscle atrophy.

1. Introduction

The muscle is an important tissue that stockpiles protein in the body; however, oxidative stress and denervation cause excessive protein degradation and induce skeletal muscle wasting [1]. Proteolytic systems are primarily responsible for eliminating contractile proteins and organelles and for shrinkage of muscle fibers [2]. In particular, two muscle-specific ubiquitin ligases, MAFbx (muscle atrophy F-box) and MuRF1 (muscle-specific RING finger protein 1), cause proteasomal degradation in skeletal muscle and contribute to muscle atrophy [3,4]. MAFbx and MuRF1, called atrogenes, are strongly increased during muscle atrophy through activation of myogenin [5] and forkhead box protein O (FoxO) [6]. Indeed, mice lacking MuRF1 or MAFbx revealed protection from denervation-induced muscle atrophy [3]. FoxO1 transgenic mice showed markedly decreased skeletal muscle mass, and conversely, FoxO1 knockdown blocked MAFbx up-regulation [7]. In addition, inflammatory cytokines and nuclear factor κB (NF-κB) signaling pathways are critically involved in muscle atrophy [8,9] through generation of intracellular reactive oxygen species (ROS) [10]. Dysregulated ROS generation is prominently associated with severe muscle loss [11].

Extensive studies have identified that nuclear factor erythroid 2-related factor 2 (NRF2)/MAF and its cytosolic repressor, kelch-like ECH-associated protein 1 (KEAP1), are indispensable for cellular adaptation to oxidative stress [12]. Oxidative stress dissociates NRF2 from KEAP1 in the cytosol and induces nuclear localization of NRF2. Nuclear NRF2 directly binds to the antioxidant response element (ARE) within the gene promoter of many antioxidants, such as heme oxygenase-1 (HO-1) [13], glutathione S-transferase α (GSTA) 1/2 [14], and NAD(P)H:quinone oxidoreductase 1 (NQO1) [15], and activates their expression, thereby protecting from oxidative stress-induced tissue injury [16]. Despite the key role of NRF2 in protection from oxidative stress, the importance of NRF2 in protection from injury-induced muscle atrophy remains unclear. In this study, we have examined whether NRF2 is involved in protecting skeletal muscle from denervation-induced muscle atrophy and characterized the molecular function during muscle atrophy.

2. Materials and methods

2.1. Mice

C57BL/6 WT and NRF2 KO mice were obtained from Jackson Laboratory (Bar Harbor, ME) and RIKEN BioResource Center (Tsukuba, Japan) and maintained in an animal facility of Ewha Womans University. Animal handling was performed in accordance with the institutional guidelines of and approved by the IACUC (2011-01-027 and 2012-01-035).

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2.2. Sciatic nerve injury-induced muscle wasting

Mice (12 weeks of age, male, 25–30 g) were anesthetized using a constant flow of isoflurane inhalation. A small incision was made below the hip bone, parallel to the sciatic nerve. The nerve was isolated by removing any adherent tissue and tightly ligated with 6-0 silk suture thread around one-third to one-half of the diameter of the sciatic nerve [17]. Separately, the sciatic nerve was exposed, but not ligated in sham-operated mice. The skin incisions were closed with surgical suture and wound clips. The mice were maintained up to 7 days and were sacrificed, followed by analyses of the tibialis anterior (TA) and gastrocnemius (GN) muscles [11].

2.3. Myogenic differentiation and muscle atrophy in vitro

C2C12 myoblasts (ATCC, Manassas, VA) were grown to 100% confluence and replaced with myogenic differentiation medium containing 2% horse serum (Invitrogen, Carlsbad, CA) for 6 days. Differentiated myocytes were then treated with dexamethasone (DEX; 500 μM, Sigma–Aldrich, St. Louis, MO) for 24 h to induce muscle atrophy in vitro.

2.4. Immunoblot analysis

Cell proteins were harvested and resolved by SDS–PAGE, followed by immunoblotting. Protein blots were incubated with antibody against myosin heavy chain (MyHC; DSHB, Iowa City, IA), FoxO1 (Cell Signaling Technology, Inc., Danvers, MA), MuRF1 (ECM Biosciences LLC, Versailles, KY), HO-1, MAFbx (Abcam, Cambridge, UK), BACH1, and β-actin (Santa Cruz Biotechnology Inc.) and subsequently with horseradish peroxidase-conjugated secondary antibody, followed by detection with enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL).

2.5. Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously [18]. Briefly, TA and GN muscles (25 mg) were finely minced in cold PBS and cross-linked with 1% formaldehyde. Muscles were homogenized and immnunoprecipitated using FoxO1 antibody or control IgG. The input DNA and eluted DNA were used for quantitative PCR and real time-PCR. Primers for HO-1 promoter were as follows: HO1-ChIP-FWD 5′-tgtgccatcactacccagaa-3′ and HO1-ChIP-REV 5′-agcagggtaaggcttggaat-3′. Relative level was calculated after normalizing to the input samples.

2.6. Total RNA isolation and real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) and used for real-time PCR (Applied Biosystems, Foster City, CA). Relative expression level was determined after normalization with GAPDH levels. The primer sets used for real time-PCR were as follows: GAPDH, 5′-tccaccttcgatgccgggg-3′ and 5′-agcgctattcattgtcatacc-3′; GSTA, 5′-ggagattgatgggatgaagc-3′, 5′-aacacctttttcaaaggcagg-3′; HO-1, 5′-accgccttcctgctcaacattgag-3′, 5′-tgttcctctgtcagcatcacc-3′; IL-1β, 5′-gtgtgccgtctttcattacacag-3′, 5′-ggacagaatatcaaccaagtgata-3′; IL-6, 5′-gaggataccactcccaacaga-3′, 5′-aagtgcatcatcgttgttcataca-3′; MAFbx, 5′-aaggctgttggagctgatagca-3′, 5′-cacccacatgttaatgttgcc-3′; MuRF1, 5′-tgaccacagagggtaaag-3′, 5′-tgtctcactcatctccttcttc-3′; myogenin, 5′-ccagcccatggtgcccagtg-3′, 5′-gcgtctgtagggtcagccgc-3′; and NQO1, 5′-gacatgaacgtcattctctg-3′, 5′-ctagctttgatctggttgtc-3′.

2.7. Reporter gene assay

293T cells were transiently transfected with mouse HO-1 promoter-linked reporter gene (pHO4.3k-luc) [19] together with NRF2, FoxO1, and/or BACH1 expression vector. pCMV (Invitrogen) encoding β-galactosidase was cotransfected as an internal control. Cell extracts were harvested 48 h after transfection and assayed using the Luciferase Assay kit (Promega, Madison, WI), and the Galacto-Light β-galactosidase assay kit (Applied Biosystems) according to the manufacturer's instructions.

2.8. Immunohistochemistry

TA muscles were fixed with 10% neutralized formalin for 24 h and embedded in paraffin. Tissue sections (5 μm thick) were prepared and stained with hematoxylin and eosin (HE) solution (Sigma–Aldrich, St. Louis, MO) and antibody against MyHC, NRF2, or FoxO1 (Cell signaling), followed by incubation with fluorescence tagged IgG or peroxide/DAB (Vector Laboratories, Burlingame, CA, USA).

2.9. Transfection of siRNA

Fully differentiated myocytes from C2C12 cells were transfected with siRNA using HiPerFect transfection reagents (Qiagen, Hilden, Germany). Transfected cells were additionally treated with Dex for 24 h. At least three different sets of siRNA for FoxO1 and HO-1 were synthesized (Bioneer, Daejeon, South Korea): siCon, 5′-ccuacgccaccaauuucgu-3′; siFoxO #1, 5′-ccagaccaggauaauuggu-3′; siFoxO #2, 5′-cuaaugugucucaaacauu-3′; siFoxO #3, 5′-caucuaugcagccuuguuu-3′; siHO-1, 5′-cagaucagcacuagcucau-3′; siHO-2, 5′-caccaaggagguacacauc-3′; and siHO-3, 5′-ccugaaucgagcagaacca-3′.

2.10. Rotarod test

Mice ($n = 12$) were divided into two groups and injected with either vehicle or hemin (25 mg/kg) for 10 days. The latency time it takes the mouse to fall off the rod rotating was measured under continuous acceleration conditions (4, 7, 10, and 20 rpm for 2 min) at days 8, 9, 10, 11 and 12 after injection. The average latency time of each group is expressed as mean ± S.E.M. for 6 mice.

2.11. Statistical analyses

Data are presented as mean ± S.E.M. and analyzed by the unpaired Student's *t*-test.

3. Results

3.1. Sciatic nerve injury induces muscle damage with an increase of NRF2 and FoxO1

To characterize the molecular mechanisms involved in muscle atrophy, we performed sciatic nerve injury and examined the muscle phenotypes. The weights of GN and TA muscles were significantly decreased and MyHC expression decreased at day 7 after nerve injury (Fig. 1A and B). However, the expression of antioxidants, such as heme oxygenase-1 (HO-1) and catalase was increased (Fig. 1B). Furthermore, the expression of atrogenes, inflammatory cytokines, and myogenin was significantly increased during muscle atrophy (Fig. 1B and C). Simultaneously, NRF2 and FoxO1 expression, but not NF-κB p65, were significantly augmented in atrophied muscle and particularly enriched in the nucleus of the muscle (Fig. 1D and E).

3.2. NRF2 is not required for induction of muscle atrophy markers and HO-1

Because NRF2 expression is important for protection against oxidative stress by the induction of antioxidants, we examined

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