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TLR2 deficiency attenuates skeletal muscle atrophy in mice

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ABSTRACT

Oxidative stress and inflammation are associated with skeletal muscle atrophy. Because the activation of toll-like receptor (TLR) 2 induces oxidative stress and inflammation, TLR2 may be directly linked to skeletal muscle atrophy. This study examined the role of TLR2 in skeletal muscle atrophy in wild-type (WT) and TLR2 knockout (KO) mice. Immobilization for 2 weeks increased the expression of cytokine genes and the levels of carbonylated proteins and nitrotyrosine in the skeletal muscle, but these increases were lower in the TLR2 KO mice. Muscle weight loss and a reduction in treadmill running times induced by immobilization were also attenuated in TLR2 KO mice. Furthermore, immobilization increased the protein levels of forkhead box O 1/3, atrogin-1 and muscle ring finger 1 in the WT mice, which was attenuated in TLR2 KO mice. In addition, immobilization-associated increases in ubiquitinated protein levels were lower in the TLR2 KO mice. Immobilization increased the phosphorylation of Akt and p70S6K similarly in WT and KO mice. Furthermore, cardiotoxin injection into the skeletal muscle increased the protein levels of atrogin-1, interleukin-6, and nitrotyrosine and increased the levels of ubiquitinated proteins, although these levels were increased to a lesser extent in TLR2 KO mice. These results suggest that TLR2 is involved in skeletal muscle atrophy, and the inhibition of TLR2 offers a potential target for preventing skeletal muscle atrophy.

1. Introduction

An imbalance between the rates of protein synthesis and protein degradation results in skeletal muscle atrophy, which commonly occurs due to a prolonged period of skeletal muscle inactivity [1]. Furthermore, skeletal muscle atrophy increases fragility and may lead to disability and metabolic disruption, such as insulin resistance [2,3].

Oxidative stress and inflammation play important roles in skeletal muscle atrophy [4,5]. Inflammation and elevated levels of oxidative stress are observed in atrophic skeletal muscle induced by unloading and immobilization, and a reduction in these two conditions has been shown to prevent skeletal muscle atrophy via the inhibition of protein degradation [5–8].

Toll-like receptors (TLR) recognize molecular microbial patterns, which stimulate signaling pathways in the innate immune system [9]. Thirteen members of the TLR family have been identified in mice, and most of these are predominantly expressed by innate immune cells [10]; however, some TLR members, including TLR2, are expressed in many types of cells and tissues, including skeletal muscle [11,12]. Accumulating evidence suggests that TLR2 detects endogenous ligands derived from damaged tissues and induces sterile inflammation [13].

The activation of TLR2 increases inflammatory cytokine levels and induces lipid and protein oxidation in non-immune cells, such as intestinal epithelial cells and cardiomyocytes [14,15]. Accordingly, TLR2 deficiency suppresses the upregulation of cytokine expression and oxidative stress induced by pro-oxidants in the liver [16,17]. Although TLR2 mediates insulin resistance in skeletal

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muscle by increasing inflammation [18,19], its pathophysiological role in skeletal muscle atrophy has not been determined. Therefore, in this study, we examined the role of TLR2 in skeletal muscle atrophy using an immobilized hindlimb model with wild-type and TLR2 knockout mice.

2. Materials and methods

2.1. Animals and immobilization

Eight-week-old male C57BL/6 wild-type (WT) and TLR2 knockout (KO) mice were fed a standard chow diet and provided free access to water. This study was conducted in accordance with the guidelines for the care and use of laboratory animals issued by Yeungnam University, and all of the experimental protocols were approved by the Ethics Committee of Yeungnam University. The left hindlimbs were immobilized by stapling the feet to exploit normal dorsal tibial flexion using a Manipler AZ-35W (Mani Inc., Utsunomiya Tochihi, Japan) [20]. The contralateral right hindlimbs were used as the controls. Immobilization was maintained for 2 weeks. The mice were then anesthetized via an intraperitoneal injection of avertin (a mixture of 2,2,2-tribromoethanol and tert-amyl alcohol), and the gastrocnemius muscles were collected, weighed and stored at −80 °C.

2.2. Cardiotoxin treatment

Cardiotoxin from *Naja mossambica mossambica* (Sigma Aldrich, Saint Louis, MO, USA) was injected (100 μl of 10 μM) directly into the left tibialis anterior (TA) muscle of anesthetized mice using a 30-gauge needle. The contralateral TA muscles were injected with phosphate buffered saline as a control. The TA muscles were dissected 3 days after injection, and the non-injected right TA muscles were used as the controls. The muscles were frozen in liquid nitrogen and stored at −80 °C prior to RNA and protein extraction.

2.3. Treadmill test

The physical performance of the skeletal muscle was assessed using a treadmill test. The running times to fatigue were measured using a motorized treadmill equipped with an electric shocker plate (Columbus Instruments, Columbus, OH, USA), as previously described [7].

2.4. Western blotting

Frozen skeletal muscle was homogenized in lysis buffer, and the protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Western blotting was performed as previously described [6]. Briefly, the protein samples were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking the membranes with 5% skim milk, they were incubated overnight at 4 °C with primary antibodies. Antibodies for atrogin-1, muscle ring finger (MuRF) 1, ubiquitin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and a nitrotyrosine antibody was purchased from Millipore. All other antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). After incubating the membranes with secondary antibodies, the protein bands were visualized using a chemiluminescent detection reagent (Millipore). The signals were detected and quantified using a LAS-3000 image analyzer and Multi Gauge 3.0 software (Fujifilm, Tokyo, Japan).

2.5. Protein carbonylation

The OxyBlot Protein Oxidation Detection Kit (Chemicon, Temecula, CA, USA) was used to determine protein carbonyl groups as previously described [6]. Briefly, the muscles were homogenized in a protein extraction solution containing 50 mM DTT. The samples were derivatized, neutralized, separated using a 12% SDS-PAGE gel, and transferred to membranes. The membranes were incubated with a rabbit anti-2,4-dinitro-phenyl antibody and then with goat anti-rabbit IgG coupled to horseradish peroxidase. The carbonylated proteins were visualized using a chemiluminescent detection reagent (Millipore).

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the skeletal muscles and reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using the 7500 System and Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions, as described previously [6]. The conditions for the reactions were as follows: an initial 10 min denaturation step at 95 °C, followed by 45 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 35 s. β-actin and GAPDH were used as the loading controls. The sequences of the primers used were based on the National Center for Biotechnology Information (NCBI) nucleotide database and designed using the Primer Express Program (Applied Biosystems): β-actin (121 bp: forward, 5′-TGG ACA GTG AGG CAA GGA TAG-3′; reverse, 5′-TAC TGC CCT GGC TCC TAG CA-3′), TLR2 (101 bp: forward, 5′- CTC AGC GAA AAT CTG ATG GT-3′; reverse, 5′-TCA AAT GAT TCT GGC TCA AAA-3′), interleukin (IL)-1β (71 bp: forward 5′-GCC CAT CCT CTG TGA CTC-A-3′; reverse 5′-AGT GCA GCT GTC TAA TGG GA-3′), IL-6 (71 bp: forward, 5′-AAA TGA TGG ATG CTA CCA AAC T-3′; reverse, 5′-CCA GAA GAC CAG AGG AAA TTT T-3′), and GAPDH (101 bp: forward, 5′-CAG TGG CAA AGT GGA GAT TG-3′; reverse, 5′-CGT TGA ATT TGC CGT GAG T-3′).

2.7. Statistical analysis

All of the results are expressed as the mean ± SEM. The statistical analyses were conducted using one-way ANOVA and Scheffe's post hoc test or the unpaired Student's t-test. Statistical significance was considered for p values < 0.05.

3. Results

3.1. Muscle weights and running times

The TLR2 mRNA levels in the gastrocnemius muscle were significantly increased after two weeks of immobilization in WT mice (Fig. 1A). The initial body weights of WT and TLR2 KO mice were similar, and after immobilization, the body weights increased but remained similar between the two groups. The gastrocnemius muscle weights were similar in the contralateral control limbs of WT and TLR2 KO mice. However, immobilization significantly reduced the muscle weights in WT mice but did not affect the muscle weights in TLR2 KO mice; as a result, the muscle weights of TLR2 KO mice were significantly higher than those of WT mice (Fig. 1C). The running times until exhaustion were also higher in TLR2 KO mice compared to WT mice (Fig. 1D).

3.2. Protein synthesis and degradation

The phosphorylation of Akt and p70S6K was similarly increased by immobilization in WT and TLR2 KO mice compared to control

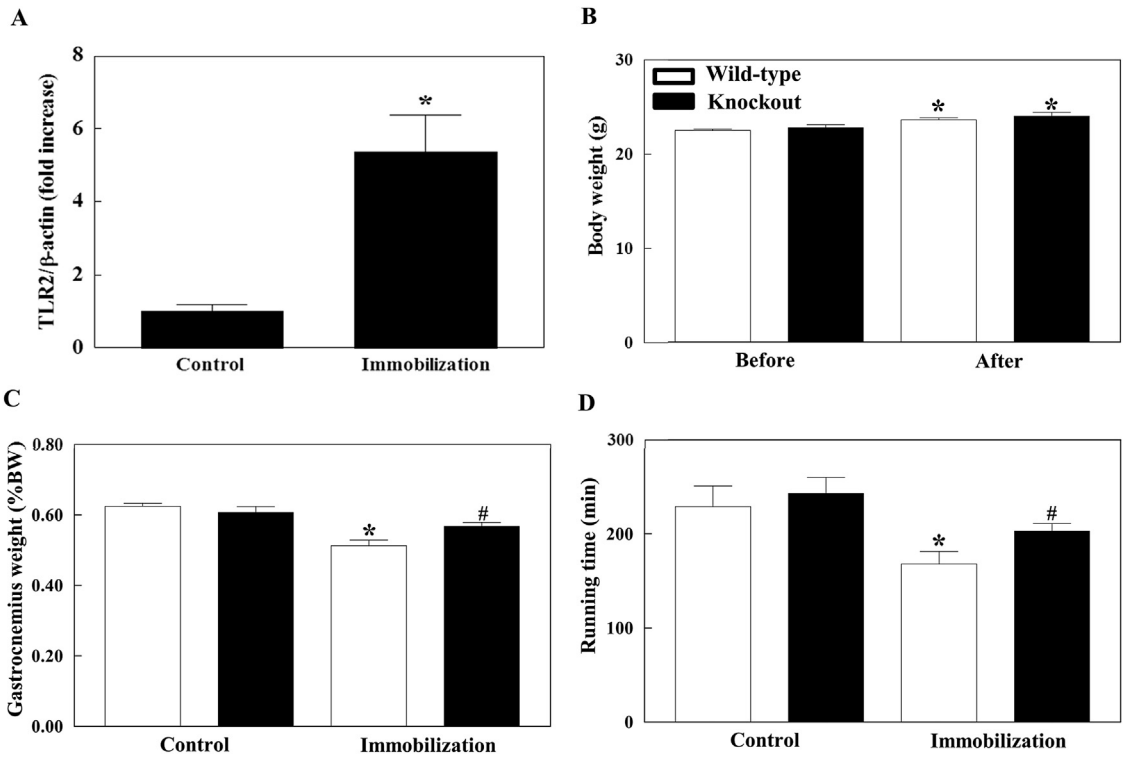

Fig. 1. Effect of TLR2 deficiency on skeletal muscle mass and function in WT and TLR2 KO mice after 2 weeks of immobilization. TLR2 gene expression in the gastrocnemius muscle of WT mice (A). Body weights (BW) before and after immobilization (B). Gastrocnemius muscle weights corrected for body weights (C). Treadmill running time until exhaustion (D). The results are expressed as the mean ± SEM for 7–11 mice per group. *$p < 0.05$ vs. controls and #$p < 0.05$ vs. immobilized WT mice.

mice (Fig. 2A–B). In the contralateral control limbs, the protein levels of forkhead box O (FOXO)1 and MuRF1 were significantly higher in the TLR2 KO mice than the WT mice. FOXO1 and FOXO3 protein levels were increased by immobilization in WT mice, whereas immobilization only increased the protein levels of FOXO1 in TLR2 KO mice. Although the protein levels of atrogin-1 and MuRF1 were increased by immobilization in WT and TLR2 KO mice, these increases were less pronounced in TLR2 KO mice (Fig. 2C–F). Immobilization also significantly increased the levels of ubiquitinated proteins in WT mice but not in TLR2 KO mice; as a result, the ubiquitinated protein levels in TLR2 KO mice were significantly lower than those in WT mice (Fig. 2G).

3.3. Cytokine expression and oxidative stress

The gene expression levels of IL-6 and IL-1β in the gastrocnemius muscle were lower in TLR2 KO mice than in WT mice in the contralateral control limbs. Cytokine expression was increased by immobilization in both WT and TLR2 KO mice, but this increase was significantly less in TLR2 KO mice (Fig. 3A–B). Immobilization also significantly increased the carbonylated protein levels in WT mice, whereas these levels were not significantly altered in TLR2 KO mice (Fig. 3C). The nitrotyrosine levels were also increased by immobilization in both the WT and TLR2 KO mice, but this increase tended to be lower in the TLR2 KO mice (Fig. 3D).

3.4. Protein degradation by cardiotoxin

Because β-actin mRNA levels were altered by treatment with cardiotoxin (data not shown), the TLR2 mRNA levels were standardized using GAPDH in cardiotoxin-injected TA muscles. Cardiotoxin treatment also increased the TLR2 mRNA levels in WT mice (Fig. 4A). The levels of IL-6 protein and nitrotyrosine were increased by cardiotoxin in both WT and TLR2 KO mice, although these increases were lower in TLR2 KO mice (Fig. 4B–C). Cardiotoxin increased the protein levels of atrogin-1 in WT mice, but not in TLR2 KO mice (Fig. 4D). Cardiotoxin significantly increased the ubiquitinated protein levels in WT and TLR-2 KO mice, but to a lesser extent in TLR2 KO mice; as a result, the levels of ubiquitinated proteins in TLR2 KO mice were lower than those in WT mice ($p < 0.06$; Fig. 4E).

4. Discussion

The present study demonstrates that immobilization induces skeletal muscle atrophy and reduces skeletal muscle function, and these changes are accompanied by increased TLR2 expression and protein degradation. Furthermore, TLR2 deficiency attenuates skeletal muscle atrophy and reduction in muscle function, which is associated with the suppression of protein degradation. In addition, cardiotoxin-induced protein ubiquitination was attenuated in TLR2 KO mice. These results indicate that TLR2 deficiency prevents skeletal muscle atrophy.

Protein degradation in skeletal muscle is regulated by lysosomal, Ca^{2+}-dependent, caspase-dependent, and ubiquitin-proteasome-dependent pathways [21]. Of these pathways, the ubiquitin proteasome-dependent pathways are mainly involved in immobilization-induced muscle atrophy [22,23]. Although the coordinated actions of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligases (E3) are required for the ubiquitin-proteasome pathway, ubiquitin ligases, such as atrogin-1 and MuRF1, are critical for tagging proteins with ubiquitin [23–25]. The protein levels of these two ubiquitin ligases are increased during immobilization-induced skeletal muscle atrophy

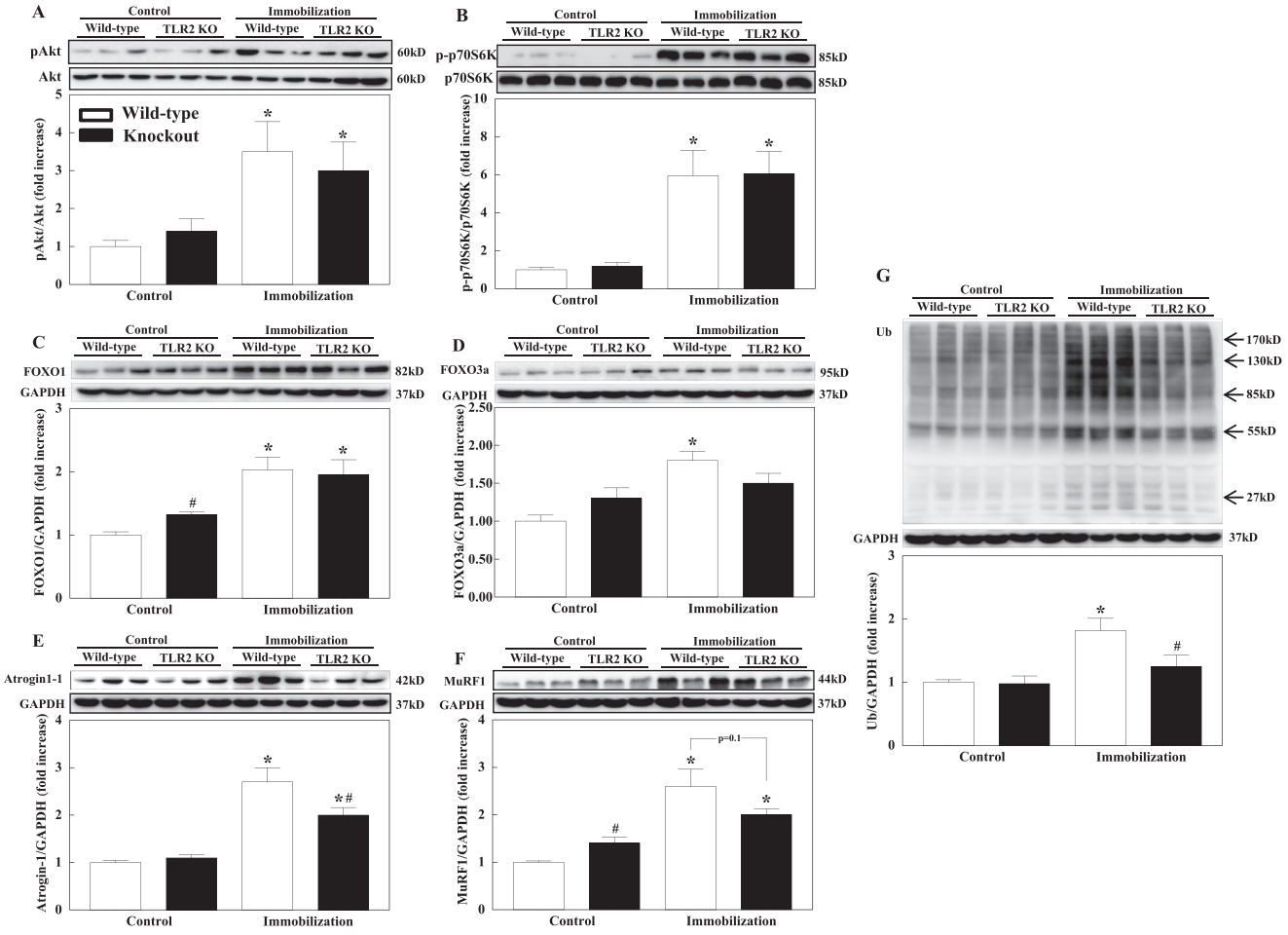

Fig. 2. Effect of TLR2 deficiency on protein synthesis and protein degradation in the gastrocnemius muscles of WT and TLR2 KO mice after 2 weeks of immobilization. Phosphorylation of Akt (A) and p70S6K (B). Protein levels of FOXO1 (C), FOXO3 (D), atrogin-1(E) and MuRF1 (F). Ubiquitinated protein level (G). The results are expressed as the mean ± SEM for 7–11 mice per group. *p < 0.05 vs. controls and #p < 0.05 vs. immobilized or non-immobilized WT mice.

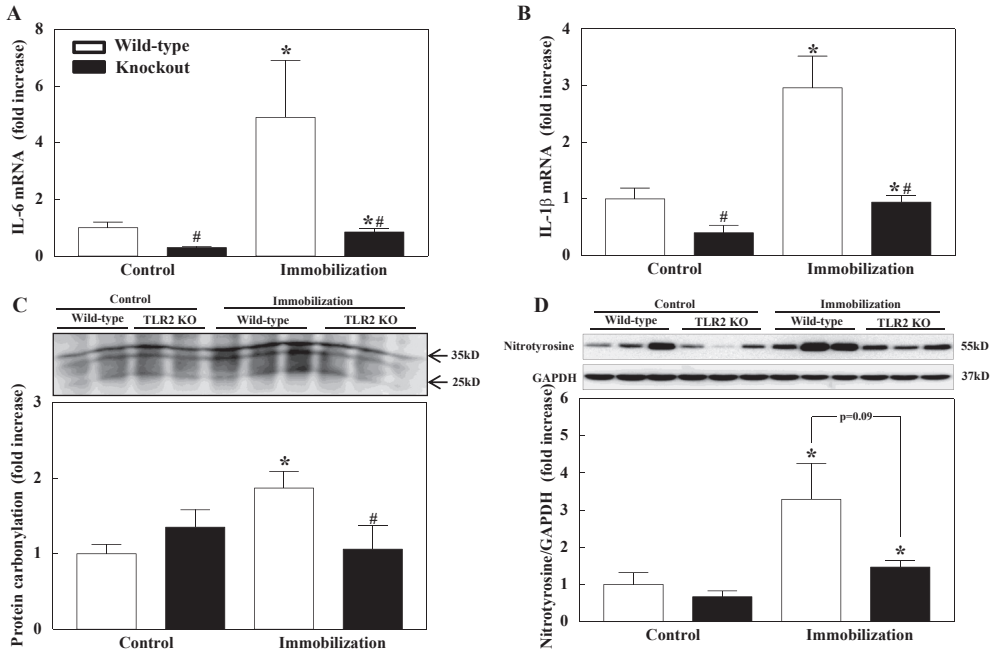

Fig. 3. Effect of TLR2 deficiency on inflammation and oxidative stress in the gastrocnemius muscles of WT and TLR2 KO mice after 2 weeks of immobilization. Gene expression of IL-6 (A) and IL-1β (B). Carbonylated protein level (C). Nitrotyrosine level (D). *p < 0.05 vs. controls and #p < 0.05 vs. immobilized or non-immobilized WT mice.

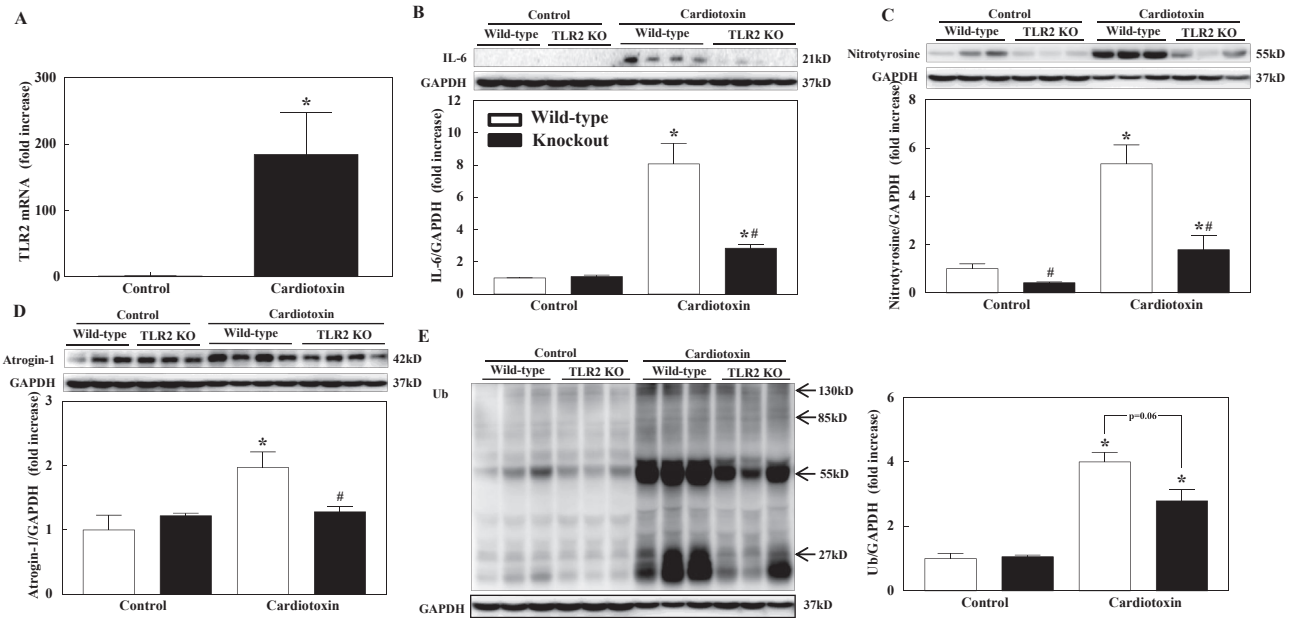

Fig. 4. Effect of TLR2 deficiency on protein degradation in the tibialis anterior muscles of WT and TLR2 KO mice after an intramuscular cardiotoxin injection. TLR2 gene expression in WT mice (A). Protein level of IL-6 (B). Nitrotyrosine level (C). Protein level of atrogin-1 (D). Ubiquitinated protein level (E). *$p < 0.05$ vs. controls and #$p < 0.05$ vs. immobilized or non-immobilized WT mice.

[6]; the overexpression of atrogin-1 results in skeletal muscle atrophy, whereas the deletion of the atrogin-1 or MuRF1 gene protects against atrophy [26]. The expression of atrogin-1 and MuRF1 is regulated by FOXO transcription factors, especially FOXO1 and FOXO3 [27,28]. Indeed, the protein levels and activities of FOXO1 and FOXO3 are increased in the atrophic skeletal muscles of mice [29,30]. Furthermore, the overexpression of FOXO proteins was shown to increase atrogin-1 mRNA levels and induce skeletal muscle atrophy, whereas FOXO deficiency prevented skeletal muscle atrophy [27,31]. Consistent with these results, we found that immobilization increased the protein levels of FOXOs and E3 ligase in immobilized skeletal muscle, which was accompanied by increased protein ubiquitination and reduced muscle mass. These results suggest that the ubiquitin-proteasome pathway is activated in immobilized skeletal muscles, which leads to skeletal muscle atrophy.

Oxidative stress and inflammation are closely linked with skeletal muscle atrophy. Immobilization is accompanied by oxidative stress and inflammation, and treatment of mice with antioxidants, such as hemin, and iNOS deficiency attenuates skeletal muscle atrophy [6,7]. Additionally, superoxide dismutase-deficient mice exhibit enhanced reactive oxygen species (ROS) generation and skeletal muscle atrophy [32], whereas superoxide dismutase overexpression attenuates skeletal muscle atrophy [33]. Recently, in addition to cytosolic ROS [34], mitochondrial ROS generation was shown to be enhanced in immobilization-induced skeletal muscle atrophy, and the treatment with antioxidants targeting to mitochondrial ROS reduced atrophy [35,36]. Oxidative stress and inflammation are associated with increased E3 ligase expression, which accelerates protein ubiquitination and skeletal muscle atrophy [4,35,37]. In the present study, immobilization increased oxidative stress and cytokine expression. We suggest that increased oxidative stress and inflammation upon immobilization activate the protein-ubiquitination pathways, thus leading to skeletal muscle atrophy. This hypothesis is supported by the observation that reduced oxidative stress and inflammation were associated with attenuated skeletal muscle atrophy in TLR2 KO mice.

The activation of TLR2 induces oxidative stress and inflammation [38], which suggests that increased TLR2 expression upon immobilization induces oxidative stress and inflammation, which in turn up-regulates MuRF1 and atrogin-1 and leads to skeletal muscle atrophy. This hypothesis is supported by our observation that TLR2 expression was increased in the immobilized muscle. Furthermore, TLR2 deficiency diminished immobilization-induced oxidative stress and inflammation, which was accompanied by reduced E3 ligase expression and muscle atrophy. Moreover, TLR2 deficiency prevented the reduction in muscle function caused by immobilization. This study demonstrates for the first time that TLR2 is involved in immobilization-induced skeletal muscle atrophy.

We also observed similar results in cardiotoxin-injected muscles. Cardiotoxin is a cobra venom toxin that exhibits cytotoxicity and depolarization effects on muscle cells [39]. When administered via injection, cardiotoxin induces severe oxidative stress and inflammation [40]. In the present study, TLR2 deficiency mitigated the oxidative stress and inflammation induced via cardiotoxin injection and resulted in reduced protein ubiquitination. These results support the conclusion that TLR2 deficiency mitigates protein ubiquitination. Although the mechanism underlying increased TLR2 expression was not determined in the present study, it is possible that endogenous ligands liberated by immobilization- or cardiotoxin-induced tissue damage stimulate TLR2 expression.

Skeletal muscle synthesis is regulated by the Akt/p70S6K pathways, and enhanced activities of Akt/p70S6K induce skeletal muscle hypertrophy in mice [41], whereas the inhibition and depletion of Akt/p70S6K result in thinner myotubes and a blunted hypertrophic response to growth factors [42]. Interestingly, the phosphorylation of Akt and P70S6K was increased by immobilization, which may suggest that protein synthesis was enhanced to counteract the increased protein degradation. In previous studies, inconsistent results were obtained regarding protein synthesis in various types of atrophy. Starvation reduces protein synthesis and increases protein degradation [43], whereas protein synthesis is somewhat increased in denervation-induced atrophic skeletal

muscle [44]. A previous study also reported reduced protein synthesis in cast-immobilized mice [6], which suggests that even changes in the type of immobilization can result in inconsistent results. Although these diverse responses cannot be explained, they do demonstrate that the signaling pathways regulating protein synthesis are highly susceptible to the experimental conditions. In the present study, although TLR2 deficiency suppressed E3 ligase and protein ubiquitination, it did not affect signaling pathways governing protein synthesis. Moreover, our result is consistent with previous findings showing that antioxidant-induced suppression of oxidative stress and inflammation does not affect signaling pathways regulating protein synthesis, although these changes may alleviate the reduction of protein degradation [6,7].

In summary, TLR2 deficiency attenuated immobilization-induced skeletal muscle atrophy and reduction in muscle function via an inhibition of protein degradation. Thus, TLR2 is involved in skeletal muscle atrophy and may serve as a potential therapeutic target.

Conflict of interest

None.

Acknowledgments

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