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Original article

Avenanthramides attenuate inflammation and atrophy in muscle cells

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Abstract

Background: Chronic inflammation is an important etiologic mechanism for muscle atrophy. Oat-derived phytochemical avenanthramides (AVAs) have been shown to suppress inflammatory responses in human clinical studies and in several cell lines *in vitro*, but their role in skeletal muscle is unclear. The aim of this study was to investigate whether AVA treatment can prevent tumor necrosis factor (TNF)-α-induced muscle fiber atrophy in C2C12 cells.

Methods: We treated 70% confluent cells for 24 h with AVA. Then, TNF-α was added to cell-cultured medium. Subsequently, cells were harvested at different time points. The cells were examined using various biochemical techniques for measuring protein, messenger RNA levels, nuclear binding activity, and viability. Fluorescence microscope was used for analysis of the myotube morphology.

Results: Cells treated with TNF-α significantly increased nuclear factor κB activation, indicated by a marked decrease of IκB ($p < 0.05$) and a 6.6-fold increase in p65-DNA binding ($p < 0.01$); however, 30 μmol of AVA-A, -B, and -C treatment reduced the binding by 33%, 18%, and 19% ($p < 0.01$), respectively, compared with cells treated with TNF-α without AVA. The interleukin-6 level increased by 2.5 fold ($p < 0.01$) with TNF-α, but decreased by 24%, 32%, and 28% ($p < 0.01$), respectively, with AVA-A, -B, and -C. The interleukin-1β level also showed a 47% increase with TNF-α ($p < 0.01$), whereas this increment was abolished in all AVA-treated cells. Reactive oxygen species production was 1.3-fold higher in the TNF-α-treated group ($p < 0.01$) but not in the TNF-α + AVAs groups. Messenger RNA levels of muscle-specific E3 ubiquitin ligase atrogin-1 increased 23% in TNF-α *vs.* control ($p < 0.05$) but was decreased by 46%, 34%, and 53% ($p < 0.01$), respectively, with treatment of AVA-A, -B, and -C. Moreover, TNF-α treatment increased the muscle RING finger 1 messenger RNA level by 76% ($p < 0.01$); this change was abolished by AVAs. Cells treated with TNF-α demonstrated a reduced proliferation compared with control cells ($p < 0.01$), but this effect was not seen in TNF-α + AVAs cells. The diameter of the C2C12 myotube decreased by 28% ($p < 0.01$) with TNF-α, whereas it showed no change when AVAs were included in the cell media.

Conclusion: These results indicated that AVAs can reduce proinflammatory cytokine and reactive oxygen species production and ameliorate TNF-α-induced myotube atrophy in muscle cells.

Keywords: Atrogenes; Interleukin; NF-κB; Skeletal muscle; TNF-α

1. Introduction

Skeletal muscle atrophy is triggered by various conditions such as muscle immobilization, denervation, starvation, and cancer cachexia.[1] The major signaling pathways known to activate immobilization-induced muscle atrophy are FoxO family transcription factors, myostatin, glucocorticoids, and nuclear factor κB (NFκB).[2] Inflammation plays an important role in the cause of muscle atrophy, during which proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 are overexpressed with enhanced NFκB activation.[2,3] TNF-α binding to sarcolemmal receptors, mostly the type-1 TNF-α receptor, stimulates mitochondrial reactive oxygen species (ROS) production and transcriptional activation of 2 E3 ubiquitin ligases, the muscle RING finger 1 (MuRF-1) and muscle atrophy F-box/atrogin-1.[4–8] Targeted proteins are conjugated by ubiquitin and degraded by the 26S proteasomes.[9,10] Whereas the IGF-1/Akt/mammalian target of rapamycin signaling pathway suppresses atrogin-1 and MuRF-1 expressions, mainly owing to its ability to phosphorylate FoxO, the main activator of the 2 ligases, catabolic signals

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such as glucocorticoids can inhibit Akt, resulting in upregulations of atrogin-1 and MuRF-1.[11,12]

Avenanthramides (AVAs) are a group of diphenolic compounds found only in oats.[13] Although more than 25 AVAs have been identified, the most abundant AVAs in oats are *N*-(3',4'-dihydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (AVA-C), *N*-(4'-hydroxy-3'-methoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (AVA-B), and *N*-(4'-hydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (AVA-A), differing only by a single moiety on the hydroxycinnamic acid ring.[13] AVAs have exhibited anti-inflammatory effects by inhibiting IL-1β- and TNF-α-stimulated NFκB activation in human aortic cells and keratinocytes,[13,14] decreasing the expression of adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) in vascular endothelial cells, and suppressing the production of IL-6, IL-8, and monocyte chemoattractant protein-1.[15] Moreover, recent studies have shown that AVA supplementation in humans could attenuate exercise-induced inflammatory markers, including plasma TNF-α and IL-6 levels, NFκB activation in neutrophils, and ROS generation in monocytes.[16] Thus, AVAs seem to be capable of acting on multiple cell types and inhibiting the NFκB-induced inflammatory pathway. However, the potential effect of AVAs on muscle inflammation and the atrophy pathway has never been investigated. In the current study, we used C2C12 muscle cells to test the hypothesis that AVA treatment would suppress TNF-α-activated NFκB signaling and proinflammatory cytokine production. We further hypothesized that AVAs would protect the muscle cells from TNF-α-induced fiber atrophy.

2. Materials and methods

2.1. Cell cultures and AVAs treatments

C2C12 cells were obtained from the American Type Culture Collection (CRL-1772: Manassas, VA, USA) and cultured in Dulbecco's modified minimum essential medium (DMEM) supplemented with 20% fetal bovine serum (Gibco, Carlsbad, CA, USA) and penicillin:streptomycin solution (50 U/mL and 50 μg/mL, respectively; Gibco) at 37°C in air with a humidified atmosphere of 5% CO_2. An AVA stock solution was made in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the cultured medium was 0.05%. A total of 30 μmol synthetic AVAs (AVA-A, -B, and -C) provided by Dr. Mitchell Wise (USDA Cereal Research Laboratory, Madison, WI, USA) were treated with fresh medium, when the cells reached 70% confluence. An equivalent amount of DMSO was added to the control cells. After the AVA was treated for 24 h, recombinant mouse TNF-α (Roche, Basel, Switzerland) was added to a final concentration of 10 ng/mL for various incubation times depending on the experimental protocols.

2.2. Western blot analysis

Cytosolic and nuclear extract fractions were isolated by following the manufacturer's instructions for an NE-PER™ nuclear and cytoplasmic extraction kit (Thermo Scientific, Waltham, MA, USA). Protein content was determined by using a Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins (12 μg) were resolved by 12% SDS-PAGE gel electrophoresis and transferred to PVDF membranes. They were then incubated with appropriate antibodies and visualized by using the electrochemiluminescence (ECL) method (Millipore, Burlington, MA, USA). The antibodies used were anti-IκBα (#9242), anti-p65 (#8242; both from Cell Signaling Technology, Danvers, MA, USA), anti-α-tubulin (loading control, ab18251), anti-histone H2B (nuclear loading control, ab1790; Abcam, Cambridge, UK).

2.3. NFκB p65 DNA binding and proinflammatory cytokines measurement

An NFκB p65 transcription factor kit (Thermo Scientific) was used to measure the p65 DNA binding levels in C2C12 myoblast whole lysates. IL-6 (BD Bioscience, Franklin Lakes, NJ, USA) and IL-1β (Thermo Scientific) protein levels were measured by the enzyme-linked immunosorbent assay method, following the manufacturer's instructions.

2.4. Intracellular ROS measurement

C2C12 myoblasts were seeded on a 96-well plate and cultured as described previously.[17] The myoblasts were then stained with 20 μmol/L of 2'7'-dichlorofluorescin diacetate (Sigma, St Louis, MO, USA) and re-incubated at 37°C for 45 min. After washing with phosphate-buffered saline, the cells were incubated with or without 10 ng/mL mouse TNF-α for 3 h at 37°C. The fluorescent signal was read by using a Synergy H1 hybrid plate reader (BioTek, Winooski, VT, USA), with an excitation wavelength at 485 nm and emission wavelength at 535 nm.

2.5. RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

All RNA was extracted and purified by using RNEasy mini columns (Qiagen, Hilden, Germany). Reverse transcription was performed to synthesize cDNA by using a SuperScript® VILO cDNA Synthesis Kit and Master Mix (Invitrogen, Carlsbad, CA, USA). Real-time quantitative polymerase chain reaction analysis was performed on a step-one RT-qPCR system (Applied Biosystems, Foster City, CA, USA) using Power SYBR® Green PCR Master Mix (Applied Biosystems). Sequences of the primer for atrogin-1, MuRF-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, internal control) are as follows: atrogin-1 forward primer, 5'-CCTGCTGTTCACAGTTGCC-3', reverse primer, 5'-CCTGCTGTTCACAGTTGCC-3'; MuRF-1 forward primer, 5'-CCTGCTGTTCACAGTTGCC-3', reverse primer, 5'-CCTGCTGTTCACAGTTGCC-3'; and GAPDH forward primer, 5'-CGTCCCGTAGACAAAATGGT-3', reverse primer, 5'-TTGATGGCAACAATCTCCAC-3'.

2.6. Cell viability assay

A Vybrant® 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, USA) was used to measure the cell

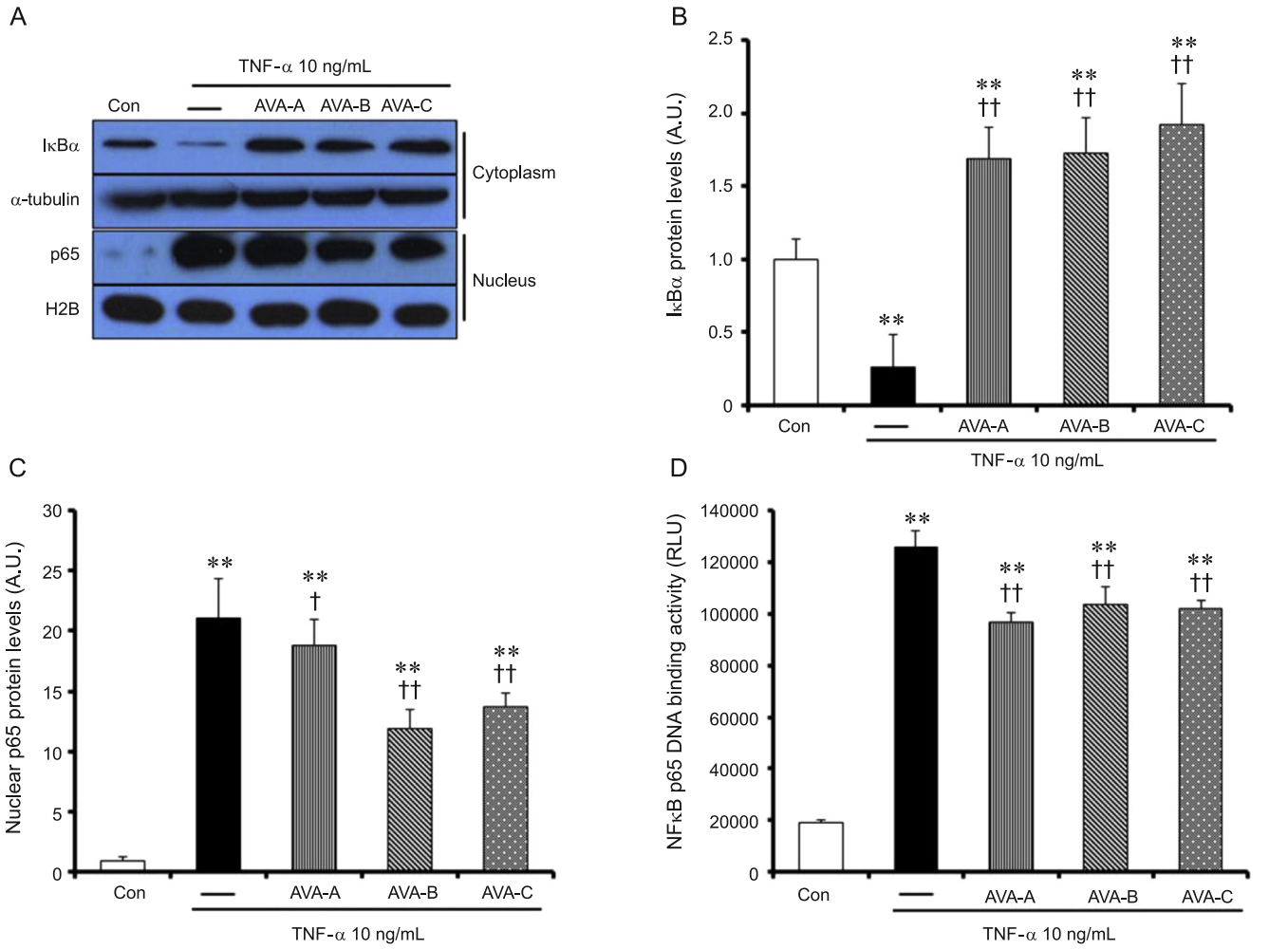

Fig. 1. Effect of AVAs on the nuclear factor κB pathway in C2C12 myoblast cells. (A) Representative Western blot images of cytoplasmic IκBα and nuclear p65. (B) The cytoplasmic protein level of IκBα. (C) The nuclear protein level of p65. (D) The level of p65 DNA binding activity. Values are mean ± SEM. $^{**}p < 0.01$, compared with control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, compared with TNF-α treatment only. AVAs = avenanthramides; Con = control; TNF-α = tumor necrosis factor-α.

viability. C2C12 myoblasts were grown in a 96-well plate and then the DMEM was replaced by a phenol red-free DMEM. To each well, 12 mmol/L of MTT stock solution was added, and the myoblasts were incubated at 37°C for 4 h. Then, 100 μL of SDS-HCl solution was added to each well, and the myoblasts were again incubated at 37°C for 4 h. Absorbance at 570 nm was read in a Synergy H1 hybrid plate reader (BioTeK).

2.7. C2C12 Myotubes diameter measurement

C2C12 myoblasts were grown as described previously. Differentiation in C2C12 myoblasts cultures was induced by 2% horse serum (Hyclone, Chicago, IL, USA) in DMEM. The images of the myotubes were taken using a digital camera mounted on a Nikon *Ti* microscope. The myotubes' diameters were measured using NIS Elements BR 3.00 software (Nikon, Tokyo, Japan). Myotube diameters were quantified in the same way they were quantified in previous studies.[18] Briefly, 5 myotubes per field were chosen randomly and measured. The average diameter of each myotube was calculated as the mean of the 3 measurements taken along the length of the myotube.

2.8. Statistical analysis

All values were represented as mean ± SEM, and statistical significance was set at $p < 0.05$. Mean values were compared between groups by one-way analysis of variance with the least significant difference method as a *post hoc* test. Data were analyzed using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of AVAs on TNF-α-induced NFκB activation

To investigate the effects of TNF-α and AVA treatment on NFκB signaling, we examined IκBα protein degradation, nuclear p65 protein levels, and p65 DNA binding activity in C2C12 myoblasts treated with either 10 ng/mL TNF-α alone or in combination with 30 μmol of AVA-A, -B, and -C (Fig. 1). IκBα protein content decreased significantly with

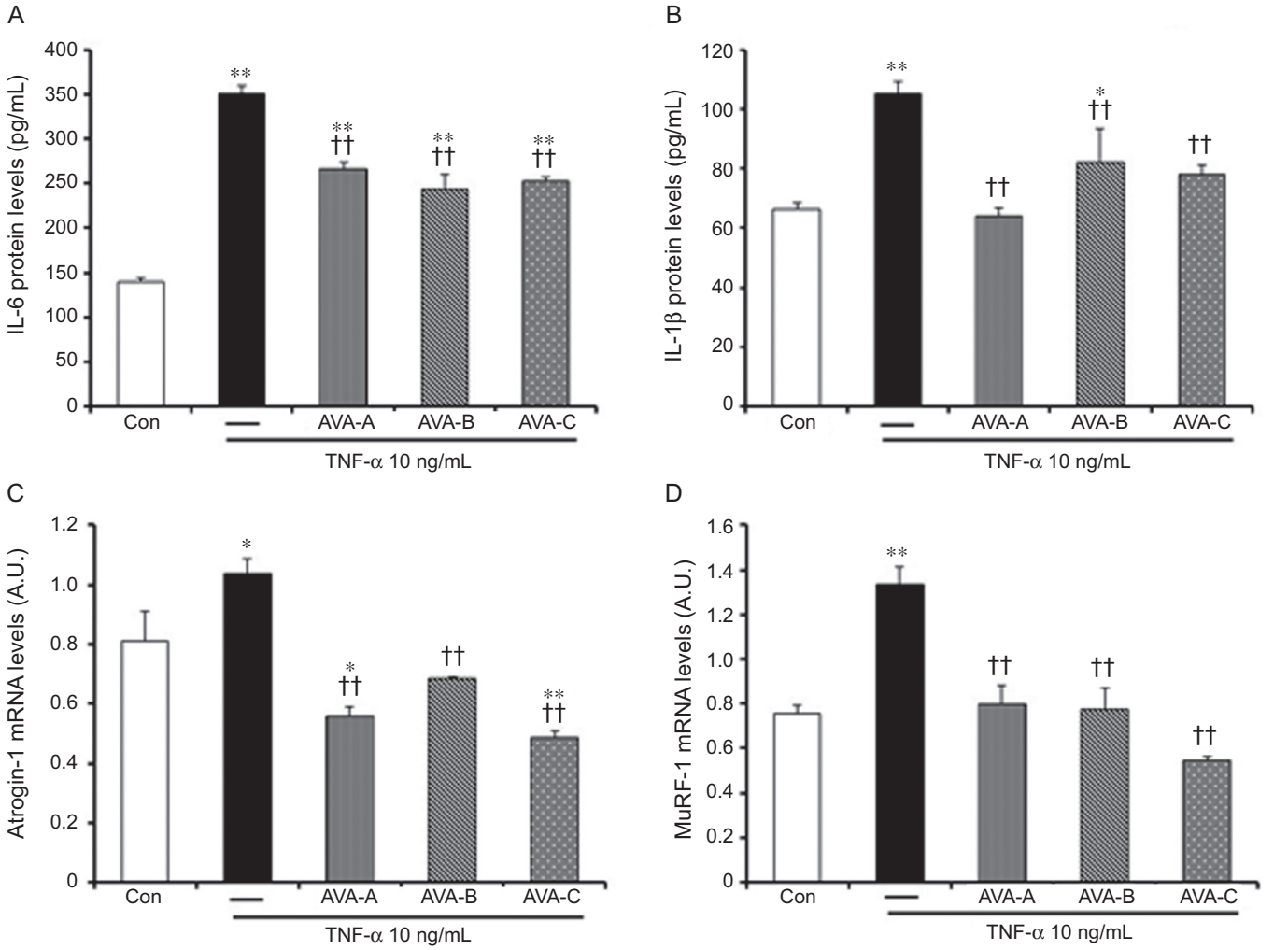

Fig. 2. Effect of AVAs on inflammatory cytokines and atrophy-related gene expressions in C2C12 myoblast cells. Protein levels of (A) IL-6 and (B) IL-1β were measured using the enzyme-linked immunosorbent assay method. Messenger RNA (mRNA) levels of (C) atrogin-1 and (D) MuRF-1 were quantified using quantitative real-time polymerase chain reaction. Values are mean ± SEM. *$p < 0.05$, **$p < 0.01$, compared with control; $^{\dagger\dagger}p < 0.01$, compared with TNF-α treatment only. AVAs = avenanthramides; Con = control; IL = interleukin; TNF-α = tumor necrosis factor-α.

TNF-α treatment ($p < 0.01$), whereas this degradation was completely abolished by pre-incubation of the cells with all forms of AVAs ($p < 0.01$; Fig. 1A, B). The nuclear p65 protein level showed a dramatic increase with TNF-α ($p < 0.01$), whereas the levels were decreased by 11% ($p < 0.05$), 43% ($p < 0.01$), and 35% ($p < 0.01$), respectively, with AVA-A, -B, and -C compared with TNF-α (Fig 1A, C). TNF-α enhanced NFκB p65 DNA binding activity by 6.6-fold compared with that of controls ($p < 0.01$). Pretreatment of the cells with AVA-A, -B, and -C decreased the activity by 33%, 18%, and 19%, respectively (all $p < 0.01$; Fig. 1A, D).

3.2. AVAs suppress TNF-α-induced proinflammatory cytokines and atrophy-related gene expression

We next investigated protein levels of the proinflammatory markers IL-6 and IL-1β as affected by TNF-α and AVA treatments. The IL-6 level was increased by 2.5-fold ($p < 0.01$) with TNF-α treatment; however, the level was decreased by 24%, 32%, and 28% (all $p < 0.01$), respectively, with AVA-A, -B, and -C from TNF-α-treated cells without AVAs (Fig. 2A). There was a 47% increase in IL-1β content with the TNF-α treatment *vs.* control ($p < 0.01$), but the level was decreased by 39%, 22%, and 26% (all $p < 0.01$) with the presence of AVA-A, -B and -C, respectively (Fig. 2B).

To determine the effect of AVA treatment on atrophy-related gene expression, we measured the messenger RNA (mRNA) levels of atrogin-1 and MuRF-1 in the various groups of cells. The atrogin-1 mRNA level was elevated by 23% with TNF-α compared with the control ($p < 0.05$); however, AVA-A, -B, and -C decreased the mRNA levels by 46%, 34%, and 53% (all $p < 0.01$), respectively, from cells treated with TNF-α alone. It is noteworthy that atrogin-1 mRNA levels in AVA-A and -C were decreased below the control level ($p < 0.05$, $p < 0.01$, respectively; Fig. 2C). Also, there was a 76% increase ($p < 0.01$) in MuRF-1 mRNA level in TNF-α-treated cells, whereas AVA-A, -B, and -C inhibited the increase by 40%, 42%, and 59% (all $p < 0.01$), respectively (Fig. 2D).

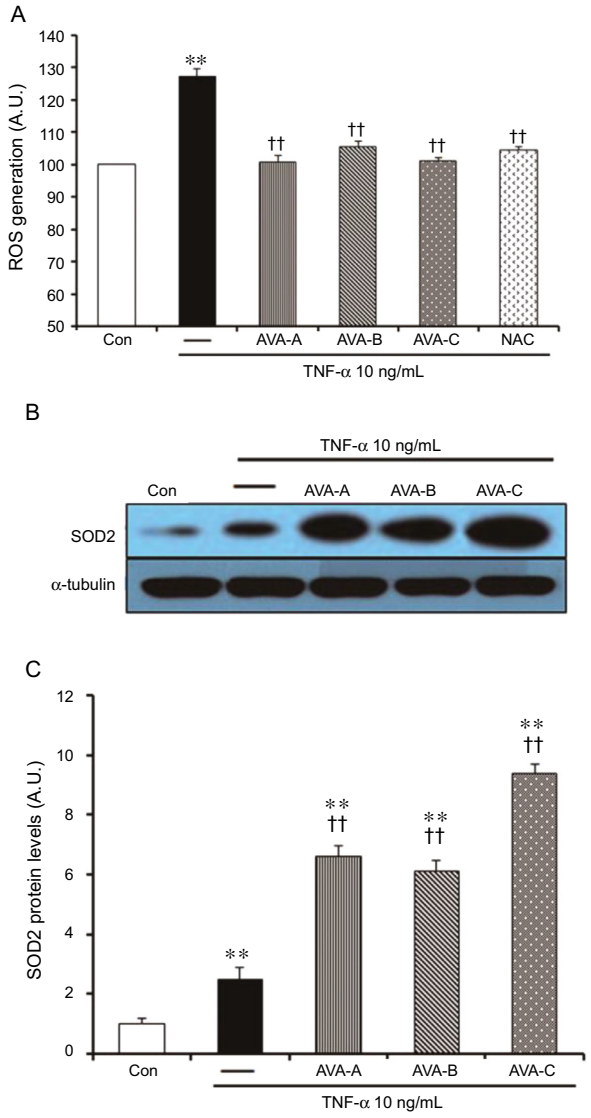

Fig. 3. Effect of AVAs on ROS and SOD in C2C12 myoblast cells. (A) ROS levels measured by 2'7'-dichlorofluorescin diacetate method. (B) Representative Western blot image of SOD2. (C) The protein content of SOD2. Values are the means ± SEM. $^{**}p < 0.01$, compared with control; $^{††}p < 0.01$, compared with TNF-α treatment only. AVAs = avenanthramides; Con = control; NAC = N-acetylcysteine; ROS = reactive oxygen species; SOD = superoxide dismutase; TNF-α = tumor necrosis factor-α.

3.3. *The effect of AVAs on ROS and superoxide dismutase (SOD)*

In cells treated with TNF-α, the intracellular ROS level was increased by 27% ($p < 0.01$) compared with control cells, but this increment was completely abolished ($p < 0.01$) with AVA-A, -B, and -C treatments (Fig. 3A). N-acetylcysteine (NAC) was purchased from sigma (Sigma-Aldrich, St. Louis, MO, USA), an antioxidant serving as a positive control, showed an inhibitory effect identical to that of AVAs ($p < 0.01$). Mitochondrial SOD2 is known to be upregulated by ROS, partly owing to activation of NFκB; therefore, we measured SOD protein content in the muscle cells. The SOD2 protein level increased by 2.5-fold ($p < 0.01$) with TNF-α treatment; interestingly, it was further increased by all fractions of AVA (6.6-fold, 6.1-fold, and 9.4-fold, respectively, with AVA-A, -B, and -C; all $p < 0.01$; Fig. 3B, C).

3.4. *Effect of AVAs on cell viability and myotube morphology*

To investigate where AVAs had a protective role in muscle cell size when the cells were exposed to TNF-α, we examined cell viability by MTT assay and myotube diameter. TNF-α treatment decreased cell viability ($p < 0.01$) in TNF-α-treated cells, but when these cells were pre-exposed with AVAs, cell viability was maintained and did not differ from that of the control (Fig. 4A). The diameter of C2C12 myotube was decreased by 28% ($p < 0.01$) in TNF-α *vs.* control, whereas AVA-B- and -C-treated cells were protected from TNF-α and showed no difference from that of the control (Fig. 4B, C).

4. Discussion

Muscle atrophy caused by denervation, immobilization, and cancer cachexia is associated with a degenerative process of muscle size and mass, mainly owing to increased protein degradation.[1] Although the initial trigger for proteolysis may vary depending on the type of atrophy, ROS production, NFκB activation, and overproduction of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β play an important role in escalating the pathogenesis and exacerbating muscle loss.[3,19] AVAs are a group of phenolic alkaloids found in oats and are known as natural anti-inflammatory and antioxidant agents.[15] Although several studies have reported that AVAs could inhibit NFκB upregulation under inflammatory and oxidative stresses,[13,14,20] it is unclear whether AVAs can also ameliorate muscle atrophy caused by cellular inflammation. Here, we demonstrate that AVAs suppressed TNF-α-induced muscle cell atrophy, along with inactivation of the NFκB pathway and reduction of proinflammatory cytokines. To our knowledge, no such data have been reported previously.

NFκB is a major redox-sensitive signaling pathway in the cell, the activation of which leads to antioxidant upregulation, inflammation, and autophagy. NFκB is activated by a variety of external stimulants, such as H_2O_2, proinflammatory cytokines (TNF-α, IL-1, and IL-6), lipopolysaccharide, and phorbol esters. These signals activate IκB kinase owing to activation of NFκB-induced kinase and protein kinase C, leading to the phosphorylation and dissociation of IκB-α, IκB-β, or IκB-ϵ, from p65 and p50, and their proteasomal degradation. As a result, p65 and p50 translocate into the nucleus and bind to the promoter of respective gene targets.[21] The NFκB pathway can be activated extrinsically upon TNF-α binding with the TNF receptor and subsequent elevation of intracellular ROS levels.[22] Our data demonstrated that the NFκB pathway was activated by TNF-α treatment in C2C12 myoblasts, as evidenced by enhanced IκB degradation and p65 DNA binding (Fig. 1). As supporting evidence of our hypothesis, all 3 forms of AVAs we tested significantly inhibited NFκB activation, shown by the lesser extent of IκB degradation

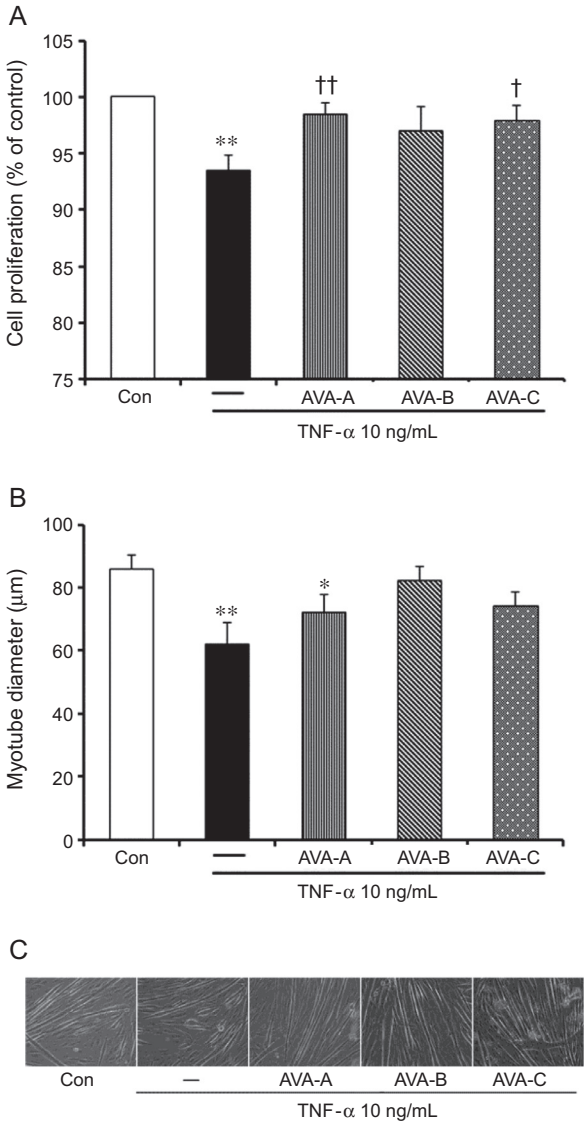

Fig. 4. Effect of AVAs on C2C12 cell viability and myotube atrophy. (A) C2C12 myoblast cell viability measured by MTT assay. (B) The diameter of the myotubes. (C) Representative images of C2C12 myotube. Values are means ± SEM. *$p < 0.05$, **$p < 0.01$, compared with control; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, compared with TNF-α treatment only. AVAs = avenanthramides; Con = control; TNF-α = tumor necrosis factor-α.

and P65 DNA binding. We further demonstrated that TNF-α-stimulated IL-6 and IL-1β upregulation was clearly attenuated (Fig. 2A, B). Because the promoter regions of IL-6 and IL-1β contain consensus p65-binding sites,[23,24] our data suggest that interference of p65 DNA binding may play a key role in accounting for the downregulation of TNF-α-induced proinflammatory cytokines. With a computerized protein-ligand docking model, we have recently discovered that AVA displays a high binding affinity with the active site of IκB kinase-α, resulting in its inactivation.[25] This finding was consistent with the report that AVAs could downregulate IL-1β-activated IκB kinase activation and proinflammatory cytokine production in human aortic cells and endothelial cells, perhaps via a common mechanism.[13]

The ROS level in the C2C12 cells was increased by TNF-α treatment, but the increase was completely abolished by the 3 forms of AVA (Fig. 3A). The mechanism by which AVAs suppress ROS generation is not clear. AVAs from oat extracts have demonstrated antioxidant properties *in vitro*.[26–28] However, it is unlikely that the flattened ROS response to TNF-α treatment can be explained by the ability of AVAs to scavenge ROS, because the concentration of AVAs in the cell medium is much lower (30 μmol/L) compared with NAC (1 mmol/L), serving as a positive control. A more plausible explanation may be related to the ability of AVAs to induce antioxidant enzymes, as has been reported in several tissues in rats after being fed an AVA-C-supplemented diet.[20] In the current study, SOD2 protein content was upregulated by TNF-α and further elevated with AVA treatments (Fig. 3B, C). This finding suggests that AVAs might have a direct role in regulating *SOD2* gene expression, thus indirectly modulating the intracellular ROS level. This unique function of AVA was not examined in the current study and requires further investigation.

In animal or human models of muscle atrophy caused by muscular disease or immobilization, there have been consistent reports that 2 muscle-specific ubiquitin ligases, atrogin-1 and MuRF-1, are upregulated, and are associated with FoxO and/or NFκB activation.[4,5,29] We were able to verify that atrogin-1 and MuRF-1 mRNA levels responded positively to TNF-α stimulation, suggesting that the signaling pathways are intact in cultured muscle cells. Prior treatment of these cells with AVAs unequivocally prevented this upregulation (Fig. 2C, D). Interestingly, AVA-C decreased ligase mRNA levels below the control group's level. Atrogin-1 and MuRF-1 gene expression are known to be controlled by anabolic pathways such as the IGF-1/AKT/mTOR axis, but this signaling mechanism is perceivably absent in the cultured myoblasts. Thus, the downregulation of atrogin-1 and MuRF-1 must be related to the decreased ROS and NFκB activation, which presumably inactivated FoxO, the major activator of ubiquitin proteolytic pathway.[2–6] However, a direct interaction between AVAs and the transactivation mechanism of these 2 atrogenes cannot be ruled out at this point. Regardless of the precise mechanism, our finding highlighted the role of AVAs in attenuating muscle protein loss via ubiquitin proteolysis.[9,10,30] Indeed, our cell viability and myotube diameter data confirmed that the TNF-α-induced muscle cell atrophy can be protected by pretreatment of AVAs.

5. Conclusion

The discovery that AVAs are effective in inhibiting NFκB activation, ROS production, and proinflammatory cytokine expression in cultured muscle cells has provided some insight into using these phytochemicals to treat muscular disorders triggered by TNF-α and inflammatory pathology.

Authors' contributions

DY, CK, and LLJ had full access to all of the data in the study and took responsibility for producing and analyzing data; DY and CK carried out the study concept and design;

DY carried out all experiments; DY and CK drafted the manuscript; LLJ revised and edited the manuscript for intellectual content; and TZ secured and provided oat AVA compounds and participated in the biochemical analysis. All the authors have read and approved the final version of the manuscript, and agreed with the order of the presentation of authors.

Competing interests

The authors declare that they have no competing interests.

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