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Conessine Treatment Reduces Dexamethasone-Induced Muscle Atrophy by Regulating MuRF1 and Atrogin-1 Expression

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Conessine, a steroidal alkaloid, is a potent histamine H3 antagonist with antimalarial activity. We recently reported that conessine treatment interferes with H_2O_2-induced cell death by regulating autophagy. However, the cellular signaling pathways involved in conessine treatment are not fully understood. Here, we report that conessine reduces muscle atrophy by interfering with the expression of atrophy-related ubiquitin ligases MuRF-1 and atrogin-1. Promoter reporter assay revealed that conessine treatment inhibits FoxO3a-dependent transcription, NF-κB-dependent transcription, and p53-dependent transcription. We also showed by quantitative RT-PCR and western blot assays that conessine treatment reduced dexamethasone-induced expression of MuRF1 and atrogin-1. Finally, we demonstrated that conessine treatment reduced dexamethasone-induced muscle atrophy using differentiated C2C12 cells. These results collectively suggest that conessine is potentially useful in the treatment of muscle atrophy.

Keywords: Conessine, muscle atrophy, MuRF1, atrogin-1, FoxO3a

Introduction

Many clinical conditions and chronic diseases such as muscle disuse, cancer cachexia, neurodegeneration, diabetes, renal failure, microgravity, and aging result in the loss of skeletal muscle mass, also called muscle atrophy [1] [2, 3]. Two E3 ubiquitin ligases, muscle RING finger (MuRF1) and muscle atrophy F-box (MAFBx/atrogin-1) are activated under skeletal muscle atrophy-inducing conditions, and MuRF1 and atrogin-1 are involved in the pathogenesis of muscle atrophy [1, 4]. Moreover, MuRF1 null mice are resistant to muscle atrophy, confirming that these ubiquitin ligases are responsible for this muscle condition [4, 5].

Thus far, many signaling pathways for muscle atrophy have been characterized. Whereas insulin-like growth factor-I suppresses the expression of MuRF1 and atrogin-1 to promote muscle growth, dexamethasone increases the expression of MuRF1 and atrogin-1 to induce muscle atrophy [5, 6]. The expression of MuRF1 and atrogin-1 appears to be regulated by two transcription factors; NF-κB and forkhead box O3a (FoxO3a) [7]. NF-κB activation induces muscle atrophy by increasing the transcription of MuRF1 mRNA, whereas FoxO3a increases the level of atrogin-1 [8, 9]. In addition, recent reports showed that p53 and p21 contribute to atrophy during limb immobilization [10].

The steroidal alkaloid conessine was originally isolated from the bark of *Holarrhena floribunda* and previous study revealed its antimalarial activity in vitro [11−13]. In addition, conessine interacts with the histamine H3 receptor and acts as a potent H3 receptor antagonist [14]. Recently, we demonstrated that conessine treatment modulates autophagic flux and interferes with hydrogen peroxide-induced cell death [15]. However, the effect of conessine on the cell signaling pathways is not fully understood.

In this report, we examined conessine-related cell signaling pathways using the promoter reporter assay, and found that conessine inhibits FoxO3a, NF-κB, and p53

signaling. These transcription factors are closely related to the expression of MuRF1 and atrogin-1. For this reason, we examined the expression of MuRF1 and atrogin-1 with conessine treatment, and found that conessine treatment represses the dexamethasone-induced expression of MuRF1 and atrogin-1. Finally, we demonstrated that conessine treatment reduces dexamethasone-induced muscle atrophy in C2C12 myotube cells. These results indicate that conessine may be a useful treatment for muscle atrophy.

Materials and Methods

Cell Culture and Myotube Formation

The human cell line HEK293A and mouse C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Korea) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic-antimycotic solution (Welgene, Korea). Both cell lines were kept at 37°C and 10% CO_2 in a humidified atmosphere incubator. For C2C12 myotube formation, 90% confluent C2C12 myoblasts were exposed to differentiation medium containing 2% horse serum (Welgene, Korea) and 1% antibiotic-antimycotic. The differentiation medium was changed every 2 days for 1 week. C2C12 myotube cells were examined using phase-contrast microscopy. Conessine and dexamethasone were obtained from the Korea Bioactive Natural Material Bank (KBNMB) and from Sigma-Aldrich (USA).

Western Blot Analysis

C2C12 mytotube cells were harvested and prepared with cell lysis buffer (50 mM HEPES, pH 7.5 (BioShop, Canada), 150 mM NaCl, 1% NP40 (Sigma Chemical Co., USA)) containing a protease inhibitor cocktail (Roche Diagnostics, Switzerland). The cells were sonicated and centrifuged at 16,100 ×*g* for 15 min at 4°C. The supernatant was stored and protein concentration in whole cell lysates was determined by Bradford assay (Bio-Rad, USA). The proteins (30 μg) were loaded onto 8−10% polyacrylamide gels in SDS-PAGE buffer and transferred to nitrocellulose membrane filters (Pall Corporation, USA). The membranes were blocked with 3% skim milk (BD Diagnostic Systems, USA) for 30 min. After that, the membranes were incubated overnight with 1:2,000 or 1:5,000 diluted primary antibody on a rocker at 4°C. Antibodies for glyceraldehyde 3-phosphate dehydrogenase and MuRF-1 were purchased from Abcam (UK). Images were obtained using a Chemidoc-it 410 imaging system (UVP, USA) and LAS4000 system (GE Healthcare, Sweden).

Quantitative Reverse Transcription PCR

Total RNA of C2C12 cells was prepared using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quality and concentration of total RNA were determined using a NanoDrop spectrophotometer (Colibri microvolume; Takara, Japan). cDNA was synthesized from 1 μg of RNA by reverse transcriptase with oligo-dT and dNTP. Quantitative reverse transcription PCR (qRT-PCR) was performed using the Step One Plus system (Applied Biosystems, USA) using SYBR green PCR master mix (Applied Biosystems, USA). qPCR reactions were conducted using the following primers: RPL4 forward 5′-ATGGCGTATCGTTTTTGGGTTGT-3′, RPL4 reverse 5′-GCT CTGGCCAGGGTGCTTTTG-3′; MuRF-1 forward 5′-TGAGGT GCCTACTTGCTCCT-3′, MuRF-1 reverse 5′-TCACCTGGTGGC TATTCTCC-3′; and atrogin-1 forward 5′-ATGCACACTGGTGCA GAGAG-3′, atrogin-1 reverse 5′-TGTAAGCACACAGGCAGGTC-3′.

Reporter Assay

For the reporter assay, HEK293 cells were cultured in 24-well cell plates in DMEM. After 18 h, the cells were transfected via lipofectamine (Invitrogen, USA). In the 24-well plates, the total plasmid DNA for the transfection was 500 ng per well, and each assay was normalized with Renilla luciferase activity. The transfected cells were lysed using Passive Lysis Buffer (Promega, USA), and were analyzed with a Dual-Luciferase Reporter Assay kit (Promega, USA).

Statistical Methods

The results of the western blot assay, reporter assay, qRT-PCR, and myotube width analysis were evaluated by a 2-tailed t test using Excel software (Microsoft, USA). $P < 0.05$ was considered significant.

Results

Signaling Pathway Screening of Conessine Treatment with Promoter Reporter Constructs

Recently, we demonstrated that conessine treatment induces autophagosome formation and also inhibits hydrogen peroxide-induced cell death [15]. These results support that conessine has the potential to treat muscle-related disease. However, the conessine-related cell signaling pathways remained unclear. For this reason, we screened the cell signaling pathways modulated by conessine treatment. HEK293 cells were transfected with luciferase constructs containing various promoters and were then treated with conessine. We used hTERT-Luc for determining telomerase activity, AP1-Luc for MAP kinase signaling, ISRE-Luc for interferon signaling, 3TP-Luc for TGFβ signaling, pOT-Luc for Wnt/β-catenin signaling, WWP-Luc for p53-dependent transcription, pELAM-Luc for the NF-κB-dependent promoter, and FHRE-Luc for FoxO3a-dependent transcription [16−22]. Whereas most reporter constructs were not affected by conessine treatment, WWP-Luc, pELAM-Luc, and FHRE-Luc showed a

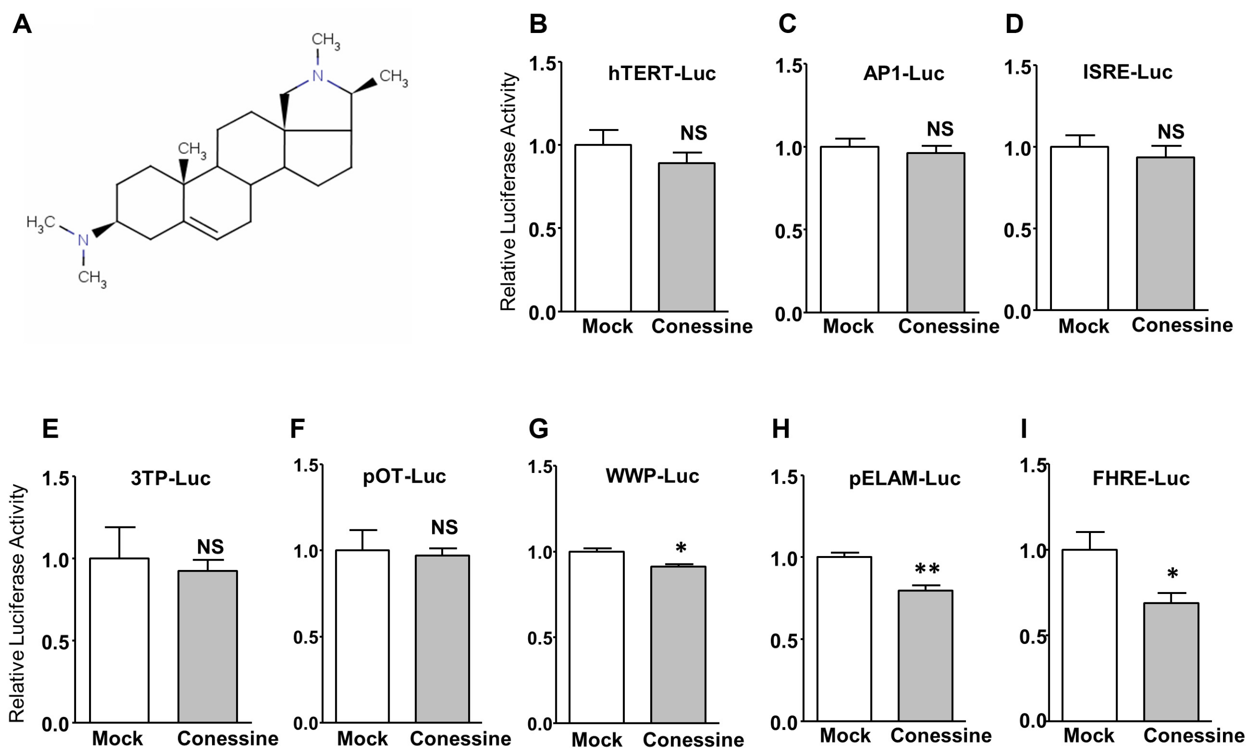

Fig. 1. Signaling pathway screening of conessine.

(**A**) Chemical structure of conessine. (**B–I**) Luciferase reporter assay with conessine. HEK293 cells were transfected with various luciferase constructs (hTERT-Luc, AP1-Luc, ISRE-Luc, 3TP-Luc, pOT-Luc, WWP-Luc, pELAM-Luc, and FHRE-Luc) and internal control plasmids (pCMV-RL). Twenty-four hours after transfection, the cells were treated with conessine (20 μM) for 24 h and luciferase reporter activity was measured. Luciferase activity was normalized with that of Renilla luciferase. The experiment was performed at least in triplicate, and the graphs show the average and standard error. Mock vs. conessine treatment, $*p < 0.05$, $**p < 0.005$; NS, not significant.

significant decrease in reporter activity (Figs. 1B–1I). These signaling pathway screening data suggest that conessine affects p53-, NF-κB-, and FoxO3a-dependent transcription.

Conessine Treatment Suppresses p53-, NF-κB-, and FoxO3a-Dependent Transcription

To confirm the screening results, we performed luciferase activity assay with various concentrations of conessine. Cells were transfected with WWP-Luc and the plasmid encoding p53 (pcDNA3/p53), and were then treated with conessine. Reporter activity was downregulated by conessine treatment in a dose-dependent manner (Fig. 2A). Next, we examined the reporter activity of pELAM-Luc in the presence of TNFα; conessine suppressed pELAM luciferase activity significantly (Fig. 2B). Finally, we confirmed pFHRE-Luc activity at various concentrations of conessine; conessine treatment significantly decreased pFHRE-Luc in a dose-dependent manner (Fig. 2C). These results confirmed that conessine treatment suppressed p53-, NF-κB-, and FoxO3a-dependent transcription.

Conessine Treatment Represses Dexamethasone-Induced Expression of MuRF1 and Atorgin-1

MuRF1 and atrogin-1 are muscle atrophy-related ubiquitin ligases [1]. Previous studies indicated that the expression of MuRF1 and atrogin-1 are mainly regulated by the NF-κB and FoxO3a pathways [7]. Because conessine treatment suppressed NF-κB-dependent transcription and FoxO3a-dependent transcription, we examined the expression of MuRF1 and atrogin-1 upon conessine treatment. First, we examined the expression of MuRF1 and atrogin-1 using qRT-PCR. We analyzed the basal expression of MuRF1 and atrogin-1 expression under conessine treatment, but we did not observe any significant change. For this reason, we administered dexamethasone, which induces the expression of MuRF1 and atrogin-1 as well as muscle atrophy [4]. When C2C12 myotube cells were treated with dexamethasone, the mRNA levels of MuRF1 and atrogin-1 were induced, and the conessine treatment decreased the expression of both MuRF1 and atrogin-1 (Fig. 3A). To confirm the RT-PCR results, we examined the protein levels of MuRF1 and

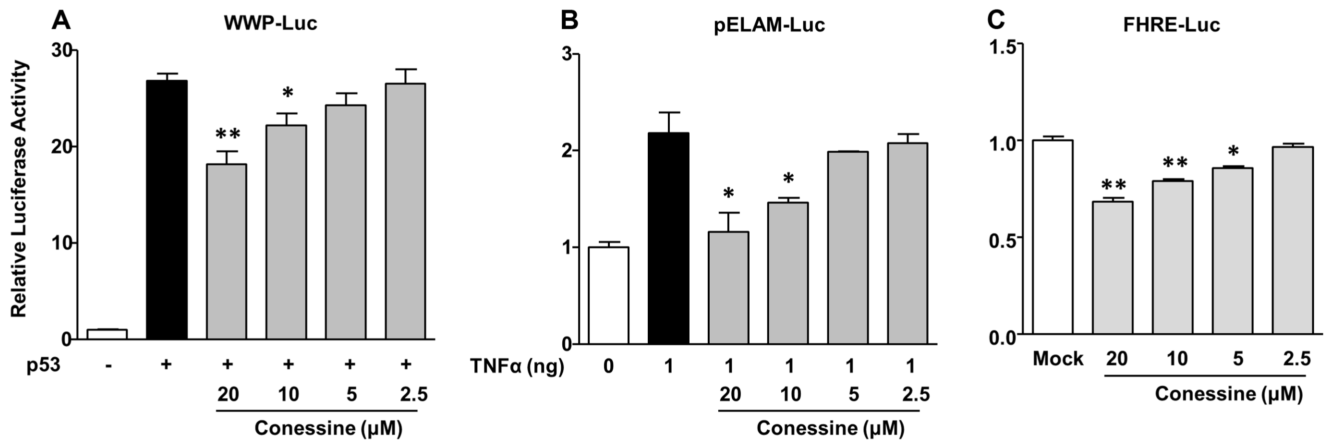

Fig. 2. Conessine treatment suppresses p53-, NF-κB-, and FoxO3a-dependent transcription.

(**A**) Conessine treatment suppressed p53-dependent transcription. HEK293 cells were transfected with WWP-Luc and the plasmid encoding p53. After 24 h, the cells were treated with the indicated concentrations (0, 2.5, 5, 10, and 20 μM) of conessine for 24 h and luciferase activity was measured. The experiment was performed in triplicate, and the graphs show the average and standard error. Control vs. conessine treatment, $^{*}p < 0.05$, $^{**}p < 0.005$. (**B**) Conessine treatment suppressed NF-κB-dependent transcription. HEK293 cells were co-transfected with pELAM-Luc. Cells were treated with conessine and TNFα (1 ng/ml). Control vs. conessine treatment, $^{*}p < 0.05$. (**C**) Conessine treatment suppressed FoxO3a-dependent transcription. HEK293 cells were transfected with FHRE-Luc and then treated with conessine. Control vs. conessine treatment, $^{*}p < 0.001$, $^{**}p < 0.0001$.

atrogin-1. We also found that the protein levels of MuRF1 and atrogin-1 were induced by dexamethasone treatment, and conessine treatment significantly decreased the protein expression of MuRF1 and atrogin-1 (Figs. 3B and 3C). These results collectively indicate that conessine decreases the expression of MuRF1 and atrogin-1.

Conessine Treatment Inhibits Dexamethasone-Induced Muscle Atrophy in C2C12 Myotubes

Since conessine treatment reduces the dexamethasone-induced expression of MuRF1 and atrogin-1, we hypothesized that conessine reduces dexamethasone-induced muscle atrophy. To test our hypothesis, we used differentiated

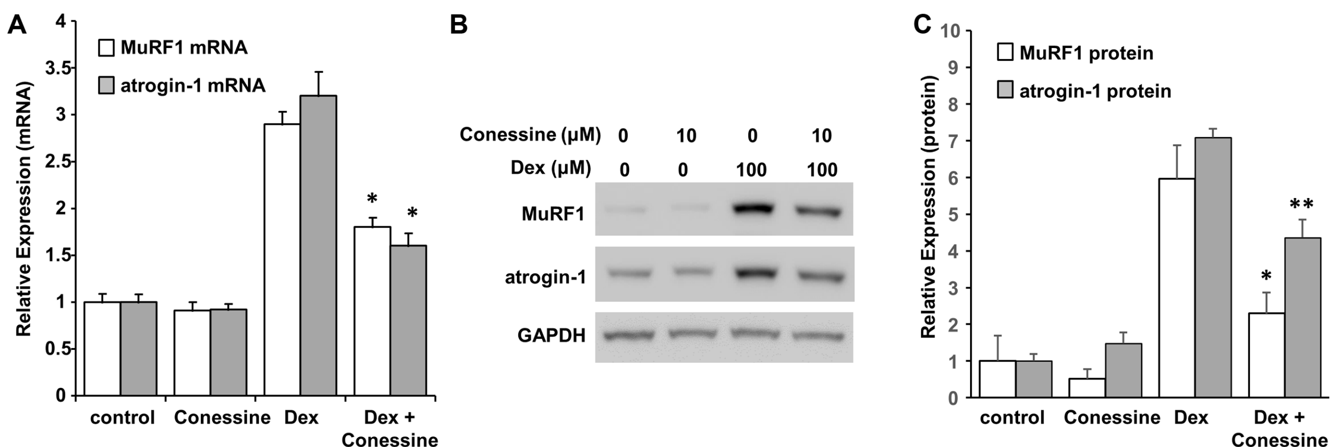

Fig. 3. Conessine treatment suppresses the expression of MuRF1 and atrogin-1.

(**A**) Conessine decreased the mRNA levels of MuRF1 and atrogin-1. C2C12 myotubes were either mock-treated or treated with dexamethasone (Dex, 100 μM), and then treated with conessine (10 μM). MuRF1 and atrogin-1 mRNA levels were analyzed via quantitative RT-PCR. Dexamethasone treatment vs. dexamethasone and conessine treatment, $^{*}p < 0.001$. (**B**) Conessine decreased the level of MuRF1 and atrogin-1 protein. The cells were collected and subjected to western blot assay using anti-MuRF1 antibody and anti-atrogin-1 antibody. (**C**) Densitometric quantification of the MuRF1 and atrogin-1 protein expression shown in (**B**). Dexamethasone treatment vs. dexamethasone and conessine treatment, $^{*}p < 0.05$, $^{*}p < 0.01$.

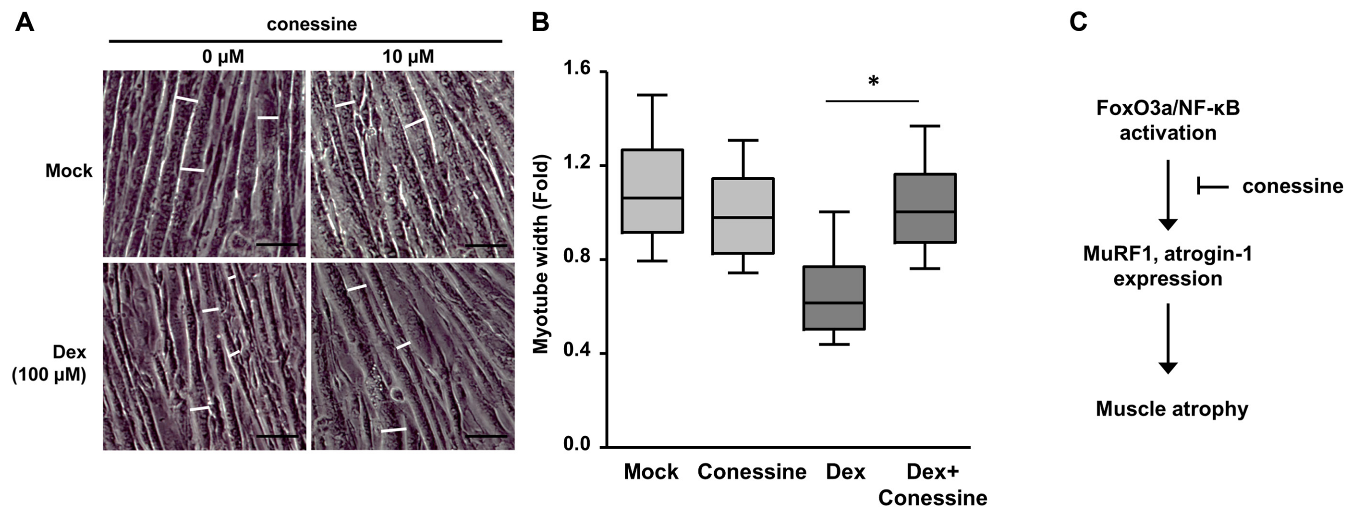

Fig. 4. Inhibitory effect of conessine on dexamethasone-induced C2C12 myotube atrophy.

(**A**) Representative photographs of C2C12 myotubes. The white bar represents the region of the C2C12 myotube used for diameter measurement. Black bars = 20 μm. (**B**) The diameter of the myotubes was measured using a high-power field with Image J. One hundred cells were measured and the graph shows diameters from 10% to 90%. Dexamethasone treatment vs. dexamethasone and conessine treatment, $*p < 0.0001$. (**C**) Schematic illustration of how conessine inhibits muscle atrophy.

C2C12 myotube cells, which were mock-treated or treated with dexamethasone for 24 h. Dexamethasone treatment decreased the diameter of C2C12 myotubes, which indicated a muscle atrophy-like phenotype (Figs. 4A and 4B). However, conessine treatment impeded the dexamethasone-induced reduction of myotube diameter (Figs. 4A and 4B). These results indicate the conessine has the potential to inhibit muscle atrophy.

Discussion

Muscle atrophy describes excessive loss of skeletal muscle mass. Because various conditions such as chronic disease and immobilization can induce muscle atrophy, diverse cell signaling pathways are involved in its induction. Here, we demonstrated that conessine is a potential therapeutic for muscle atrophy. We used the luciferase reporter assay for screening cell signaling pathways and each construct was designed to respond to the activation of specific cell signaling. Conessine treatment significantly decreased p53-, NF-κB-, and FoxO3a-dependent transcription. Based on our experimental data, the decrease in NF-κB-dependent transcription and FoxO3a-dependent transcription was marked (Figs. 2B and 2C). The reduction in p53-dependent transcription was less dramatic, although it was statistically significant (Figs. 1G and 2A). For this reason, both the NF-κB and FoxO3a pathways appear to be the major cell signaling pathways modulated by conessine treatment. Interestingly, both the NF-κB pathway and FoxO3a pathway are responsible for the expression of muscle atrophy-related ubiquitin ligases, MuRF1 and atrogin-1. Therefore, we speculate that the downregulation of the NF-κB and FoxO3a pathways by conessine will reduce muscle atrophy.

Previously, we reported that conessine treatment inhibits autophagic flux and results in the accumulation of autophagosomes [15]. Although the regulation of autophagic flux is not clearly understood, several reports support that FoxO3a activation is required for efficient autophagic flux [23, 24]. Therefore, the inhibition of FoxO3a activity by conessine may contribute to the inhibition of autophagic flux.

NF-κB has an essential role in inflammation, and dysregulation of NF-κB signaling results in various diseases such as a cancer. Thus, an NF-κB blocker is not only a potential anti-inflammatory drug, but also a potential anti-cancer drug. Conessine decreases basal NF-κB activity (Fig. 1H) as well as TNFα-induced NF-κB activity (Fig. 2B). Here, we report that conessine is a potential therapeutic for muscle atrophy; however, conessine may also have an anti-inflammatory effect and anticancer activity. Conessine blocks autophagic flux, and could thus be used to kill cancer cells requiring efficient autophagy and NF-κB activity. Further study will be required to elucidate the possibility of conessine application for other purposes.

Although the difference was not marked, conessine treatment decreased p53-dependent transcription (WWP-

Luc). p53 regulates the cell cycle by modulating p21 expression, and also induces apoptosis via pro-apoptotic genes such as *Bax*, *Puma,* and *Noxa* [25]. Thus, conessine may have a regulatory role in the cell cycle and apoptosis via regulation of p53-dependent transcription. We demonstrated previously that conessine treatment interferes with hydrogen peroxide-induced cell death, and hydrogen peroxide also activates p53 to induce apoptosis [15, 26]. For this reason, the regulation of p53 by conessine may contribute to the suppression of hydrogen peroxide-induced cell death.

In this report, we revealed that conessine reduces muscle atrophy by suppressing NF-κB and FoxO3a signaling. As NF-κB and FoxO3a are well-known transcriptional regulators of MuRF1 and atrogin-1, conessine's inhibition of NF-κB/FoxO3a-related pathways can prevent muscle atrophy via the downstream regulation of MuRF1 and atorgin-1. Further study will be needed to develop clinical applications for conessine.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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