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(Citation)
Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,271:111262

(Issue Date)
2022-09

(Resource Type)
journal article

(Version)
Accepted Manuscript

Atrogin-1 knockdown inhibits the autophagy-lysosome system in mammalian and avian myotubes

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Abstract

Atrogin-1 plays an important role in ubiquitin-proteasome proteolysis in vertebrate skeletal muscles. Recently, atrogin-1 has been shown to be involved in the autophagy-lysosome system, another proteolytic system, in the murine and fish hearts and skeletal muscles. With the aim to elucidate the effect of atrogin-1 on the autophagy-lysosome system in mammalian and avian skeletal muscles, this study has examined the effects of atrogin-1 knockdown on autophagy-lysosome-related proteins in C2C12 and chicken embryonic myotubes. Using the levels of microtubule-associated protein light chain 3 (LC3)-II protein, it was confirmed that atrogin-1 knockdown blocked the autophagic flux in both the myotubes. In addition, atrogin-1 knockdown in C2C12 myotubes significantly decreased the level of autophagy-related gene (ATG)12-ATG5 conjugate, which is supposedly necessary for the fusion of autophagosomes and lysosomes. Atrogin-1 knockdown also resulted in downregulation of forkhead box O3, a transcription factor for ATG12. These data suggest that atrogin-1 is essential for the normal autophagy-lysosome system in the striated muscles of vertebrates.

Key words: C2C12, chicken myotube, LC3, ATG

Introduction

The skeletal muscle is the most abundant tissue in the body and the major site of glucose and fatty acid expenditure (Abdul-Ghani and DeFronzo, 2010; Furler et al., 2000). Since excessive glucose and fatty acids is a cause of diabetes and obesity, maintenance of skeletal muscle mass is important for preventing such diseases (Kumar et al., 2020). In the livestock industry, skeletal muscle mass, the most important trait in meat-type breeds, is an interesting target for research. Skeletal muscle mass is controlled by the balance between protein synthesis and degradation (Sandri, 2008, 2013): skeletal muscle mass decreases when the rate of protein degradation exceeds that of protein synthesis. Numerous studies have been conducted to elucidate the mechanisms underlying protein metabolism in the skeletal muscles of various animal species.

Atrogin-1 is a muscle-specific E3 ubiquitin ligase that is an important regulator of protein degradation in the ubiquitin-proteasome system (Foletta et al., 2011; Gumucio and Mendias, 2013). Interestingly, atrogin-1 deficiency impairs autophagy and promotes cardiomyopathy in mice (Zaglia et al., 2014). Similarly, in atrogin-1-deficient fish, autophagy is severely impaired, and progressive impairment of heart and skeletal muscle function and disruption of muscle structure are observed (Bühler et al., 2016). Therefore, atrogin-1 may be necessary for normal proteolysis in the striated muscles of verterbrates. However, it remains unclear whether atrogin-1 affects the autophagy-lysosome system in the skeletal muscles of mammals and birds.

Autophagy is a major pathway for intracellular degradation (Wesselborg and Stork, 2015). To date, it has been extensively investigated and reviewed in many articles (Martens and Fracchiolla 2020; Molino et al., 2017; Wesselborg and Stork, 2015; Xie and Klionsky, 2007; Yu et al., 2015). The autophagy pathway requires the formation of autophagosomes, double-membrane vesicles containing sequestered cytoplasmic material, which ultimately fuse with lysosomes. The autophagy-related genes (ATGs) /proteins are key

players in this process. ATG8 family proteins are evolutionarily conserved ubiquitin-like modifiers that are ultimately attached to phosphatidylethanolamine of the membrane in a process referred as lipidation (Martens and Fracchiolla 2020; Molino et al., 2017; Wesselborg and Stork, 2015; Xie and Klionsky, 2007). Therefore, ATG8 protein lipidation is a hallmark of autophagy (Martens and Fracchiolla 2020). However, it remains unclear whether atrogin-1 affects lipidation-regulating factors in striated muscles of mammals.

Although autophagy is recognized as an important conserved cellular process, the role of autophagy in farm animals has received little attention (Tesseraud et al., 2021). In particular, there have been few studies that have examined the involvement of autophagy in the muscle growth and meat quality of chickens (Tesseraud et al., 2021). As typical domestic fowl, investigating the autophagy–lysosome system in the skeletal muscles of chickens could potentially lead not only to improvements in the productivity of the poultry industry but also enable us to gain a better understanding of autophagy in other avian species.

In this study, we investigated the effects of atrogin-1 knockdown on the autophagy-lysosome system in mammalian and avian myotubes using small interfering RNA (siRNA).

Materials and methods

1. Cell culture

1.1. C2C12 myotubes

As described in a previous study (Saneyasu et al., 2018), C2C12 myoblasts (DS Pharma Biomedical Co. Ltd, Osaka, Japan) were grown prior to assay in high-glucose Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% FetalClone III (GE Healthcare, Chicago, IL, US), 100 U/mL of penicillin, and 100 μg/ml of streptomycin (Nacalai Tesque, Inc., Kyoto, Japan) at 37°C and 5% CO2 in a

84 humidified air. The cells were plated at 20,000 cells/well in 12-well plates and incubated
85 until they reached confluency. To induce myoblast fusion to form myotubes, the medium
86 was replaced with DMEM supplemented with 2% horse serum (Thermo Fisher Scientific
87 Inc., Waltham, MA, US).
88 ON-TARGETplus mouse Fbxo32 siRNA and ON-TARGETplus non-targeting siRNA #1
89 were purchased from Horizon Discovery Ltd (Cambridge, UK). siRNA transfection was
90 performed using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies,
91 Carlsbad, CA, USA), according to the supplier's protocol. C2C12 myotubes were treated
92 with siRNA-containing medium for 2 days, followed by treatment with fresh medium
93 without siRNA for 1 day.
94 C2C12 myotubes treated with siRNA were starved with phosphate-buffered saline for 8
95 h in the absence or presence of 30 μM chloroquine (Nacalai Tesque, Inc., Kyoto, Japan),
96 a lysosome inhibitor, or 10 mM 3-methyladenin (Cayman Chemical Co., MI, USA), an
97 inhibitor of class III phosphatidylinositol 3-kinases. The concentrations used for these
98 inhibitors were based on reference to previous studies (Bloemberg and Quadrilatero,
99 2016; Rossi et al., 2009).
100
101 *1.2. Chicken embryonic myotubes*
102 Chicken embryonic myotubes (CEM) were formed as described previously (Saneyasu et
103 al., 2019). Breast muscles dissected from 14-day-old chick embryos were minced using
104 surgical scissors and digested with Hank's balanced salt solution (+) (Nacalai Tesque,
105 Inc., Kyoto, Japan) containing 0.2% collagenase (Worthington Biochemical Corp.,
106 Lakewood, NJ, USA) for 20 min at 37°C. Cells were collected by centrifugation and
107 resuspended in DMEM (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 15%
108 FetalClone III (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), 1× non-essential
109 amino acid solution (Nacalai Tesque, Inc., Kyoto, Japan), and 1×

gentamicin/amphotericin solution (Life Technologies, Carlsbad, CA, USA). The cell suspension was passed through a cell strainer to remove the tissue debris and transferred to an uncoated flask to allow the attachment of fibroblasts. After 1 h, the unattached cells were transferred to another uncoated flask and this procedure was repeated 3 times. Unattached cells were counted and plated onto collagen I-coated 12-well plates at a density of 1×10^5 cells/well. Cells were incubated in the medium described above at 37°C and 5% CO_2 in humidified air until myotube formation.

Chicken atrogin-1-targeting siRNA (sense, 5'-CAGCAGAACAUCAGGCUAAUU-3'; antisense, 5'-UUAGCCUGAUGUUCUGCUGUU-3') was designed using the siDESIGN Center and synthesized by Horizon Discovery Ltd. (Cambridge, UK). ON-TARGETplus non-targeting siRNA #1 was used as the control. siRNA transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA), according to the supplier's protocol. Chicken embryo mytubes were treated with siRNA-containing medium 199 supplemented with 15% FetalClone III for 3 days.

siRNA-treated CEM were starved in FetalClone III-free medium 199 for 3 h in the absence or presence of 30 μM chloroquine or 10 mM 3-methyladenin.

2. Western blot analysis

Western blot analysis was performed as previously described (Saneyasu et al., 2019). Briefly, the cells were rinsed with ice-cold phosphate-buffered saline (PBS) and scraped into 100 μL of lysis buffer containing 150 mM sodium chloride, 10 mM tris(hydroxymethyl)aminomethane, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid, 1% Triton X-100, 0.5% NP-40, 100 mM sodium fluoride, 23 mM sodium phosphate, 2 mM sodium orthovanadate, and protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). Cell lysates were ultrasonicated and centrifuged at 17,900 × g for 15 min at 4°C. The

supernatants were stored at -80°C. The protein concentration was determined using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting using HorizeBlot (ATTO Co., Tokyo, Japan), according to the manufacturer's instructions. Bands were detected using Chemi-Lumi One Super (Nacalai Tesque, Inc., Kyoto, Japan), visualized with LumiCube (Liponics Inc., Tokyo, Japan), and quantified using the CS Analyzer software (ATTO Co., Tokyo, Japan). Anti-beclin-1 (#3495), anti-phospho-adenosine monophosphate-activated protein kinase (AMPK)-α (Thr172) (#2531), anti-AMPKα (#2532), anti-Atg3 (#3415), anti-Atg7 (#8558), anti-Atg12 (#4180), anti-Atg16L1 (#8089), anti- microtubule-associated protein light chain 3 (LC3) (#12741), anti-forkhead box O3 (FOXO3) (#12829), anti-phospho-FOXO3 (Ser253) (#9466), anti-β-actin (#8457), anti-α-tubulin (#2125), horseradish peroxidase (HRP)-linked anti-rabbit IgG (#7074), and HRP-linked anti-rat IgG (#7077) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-F-box protein 32 (Fbx32) (ab168372) and anti-lysosome-associated membrane protein 2 (LAMP2) (ab13524) antibodies were purchased from Abcam (Cambridge, UK). Anti-β-actin and anti-α-tubulin antibodies were used as loading controls. Results are shown relative to those of the control group.

3. Statistical analysis

Data were analyzed using a two-tailed *t*-test performed using Excel 2013 (Microsoft, USA).

Results

Treatment with atrogin-1 siRNA significantly decreased the atrogin-1 protein levels in both C2C12 and chicken myotubes (Fig1A,2A; sFig1,2). Treatment with autophagy

inhibitors significantly increased the levels of LC3-II protein in myotubes pretreated with non-targeting siRNA, whereas no significant change was observed in atrogin-1-knockdown myotubes (Fig1B,2B; sFig1,2).

Under serum-free culture conditions without autophagy inhibitors, the treatment with atrogin-1 siRNA significantly increased the level of LC3-II protein in C2C12 myotubes (Fig3), similar to the results in fish embryos (Bühler et al., 2016). These findings raise the possibility that atrogin-1 knockdown/knockout promotes LC3 lipidation. Although no significant changes were observed in the phosphorylation rate of AMPK and the protein levels of ATG3, ATG7, ATG16L1, beclin 1, and LAMP2, the levels of the ATG12-ATG5 conjugate significantly decreased upon treatment with atrogin-1 siRNA (Fig3).

The transcription of ATG12 is controlled by FOXO3 (Zhao et al., 2007). In the present study, the protein levels of phosphorylated and total FOXO3 were significantly decreased by atrogin-1 knockdown in C2C12 myotubes (Fig3).

Discussion

Atrogin-1 expression levels are upregulated in the skeletal muscle of mammalian atrophy models such as conditions of fasting, denervation, immobilization, and aging (Bodine and Baehr, 2014). A highly positive linear correlation was observed between the rate of protein breakdown and the mRNA levels of atrogin-1 in C2C12 myotubes (Sacheck et al., 2004). Similarly, atrogin-1 expression is positively correlated with N^{τ}-methylhistidine content, a marker of myofibrillar proteolysis, in the skeletal muscles of broiler chickens (Ohtsuka et al., 2011). In addition, the mRNA levels of atrogin-1 are lower in the skeletal muscles of commercial broiler chickens than in those of other strains, such as the red jungle fowl, leghorn chickens, and Chinese local chickens (Li et al., 2019). Therefore, the inhibition of atrogin-1 expression appears to be one of the strategies to increase skeletal muscle weight in vertebrates. However, atrogin-1 knockout significantly decreases

188 muscle weight in old mice (Sandri et al., 2013). Moreover, atrogin-1 deficiency leads to
189 myopathy via impaired autophagy in the murine heart (Zaglia et al., 2014) and fish heart
190 and skeletal muscle (Bühler et al., 2016). The present study also suggests that the
191 downregulation of atrogin-1 expression impairs autophagy in mammalian and chicken
192 skeletal muscles. In contrast, chronic cardiomyocyte-specific overexpression of atrogin-
193 1 reduces aging-related cardiac fibrosis in mice (Mota et al., 2018). Therefore, it is likely
194 that chronic inhibition of atrogin-1 expression leads to the loss of striated muscle in
195 vertebrates.
196 A previous study identified charged multivesicular body protein 2B (CHMP2B) as a target
197 of atrogin-1 (Zaglia et al., 2014). CHMP2B is a component of the endosomal sorting
198 complex required for transport III, and its dysfunction leads to the accumulation of
199 autophagosomes due to the inhibition of autophagosome-lysosome fusion (Filimonenko
200 et al., 2007; Lee et al., 2007; Rusten et al., 2007). Therefore, autophagosome-lysosome
201 fusion is compromised in atrogin-1 knockout hearts (Zaglia et al., 2014). However,
202 another study reported an increase in CHMP2B protein levels in atrogin-1
203 cardiomyocyte-specific transgenic mice (Mota et al., 2018). These findings raise the
204 possibility of other factors being involved in the impairment of autophagy caused by
205 atrogin-1 deficiency. A previous study in mammalian cells revealed that the
206 autophagosome maturation mechanism is mediated by tectonin beta-propeller repeat
207 containing 1 (TECPR1) and ATG12-ATG5 conjugate (Chen et al., 2012). TECPR1
208 promotes autophagosome-lysosome fusion by associating with both ATG12-ATG5
209 conjugate and phosphatidylinositol 3-phosphate (PtdIns(3)P), and ATG12 is required for
210 PtdIns(3)P binding of ATG5-TECPR1 (Chen et al., 2012), suggesting that ATG12 is
211 required for the fusion of autophagosomes and lysosomes. In the present study, atrogin-1
212 knockdown significantly increased the levels of LC3-II, while decreasing the levels of
213 ATG12-ATG5 conjugate and FOXO3 proteins in C2C12 myotubes (Fig 3). These

findings suggest the possible mechanism by which atrogin-1 knockdown inhibits FOXO3-mediated expression of ATG12, resulting in impaired autophagy due to compromised fusion of autophagosomes and lysosomes in mammalian skeletal muscles. In the present study, we found that atrogin-1 knockdown promoted an increase in the levels of LC3-II protein in C2C12 myotubes (Fig. 3), which is consistent with the findings of previous studies on fish embryos (Bühler et al., 2016) and murine hearts (Zaglia et al., 2014). However, we detected non-significant changes or significant reductions in the assessed LC3 lipidation-related factors (Fig. 3), which accordingly tends to indicate that atrogin-1 does not play a role in LC3 lipidation, or at least does not promote this process. Atrogin-1 also functions as a coactivator of FOXO1/3 via ubiquitylation (Li et al., 2007), and overexpression of atrogin-1 in rodent cardiomyocytes and hearts has been observed to induce a reduction in the phosphorylation of FOXO3 (Li et al., 2007; Mota et al., 2018). Although in the present study, we failed to detect any significant changes regarding the phosphorylation of FOXO3 in C2C12 myotubes (data not shown), we did observe significant reductions in the levels of phosphorylated and total FOXO3 proteins in response to atrogin-1 knockdown (Fig, 3). These findings thus indicate that atrogin-1 controls not only the transcriptional activity of FOXO3 but also levels of FOXO3 protein in striated muscles. Further studies are required to clarify how atrogin-1 regulates FOXO3 protein expression.

Conclusion

The present study suggests that atrogin-1 is essential for the normal autophagy-lysosome system in the skeletal muscles of mammals and birds, and that atrogin-1 may be involved in the autophagy-lysosome systems by regulating the FOXO3 and ATG12 protein levels in mammalian skeletal muscles.

ACKNOWLEDGMENT

This work was supported by Kieikai Research Foundation. We would like to thank Editage (www.editage.com) for English language editing.

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337
338
339 **Figure legends**
340 **Figure 1. Effects of atrogin-1 small interfering RNA and autophagy inhibitors on the**
341 **levels of atrogin-1 and microtubule-associated protein light chain 3 (LC3)-II protein,**
342 **respectively, in C2C12 myotubes**. Data are expressed as the mean ± standard error of
343 the mean (SEM) in each group (n = 6). The two-tailed *t*-test was used to analyze the

differences between the groups. **Significance with respect to the non-targeting (NT) or phosphate-buffered saline (PBS) group ($P < 0.01$). 3-MA, 3-methyladenine; Cq, chloroquine; NT, non-targeting siRNA; siAtr, atrogin-1 siRNA; PBS, phosphate-buffered saline.

Figure 2. Effects of atrogin-1 siRNA and autophagy inhibitors on the levels of atrogin-1 and LC3-II protein, respectively, in chicken embryonic myotubes. Data are expressed as the mean ± SEM in each group (n = 6). The two-tailed *t*-test was used to analyze the differences between the groups. **,*,Significance with respect to the NT or PBS group ($P < 0.01$ and $P < 0.05$, respectively). 3-MA, 3-methyladenine; Cq, chloroquine; NT, non-targeting siRNA; siAtr, atrogin-1 siRNA; PBS, phosphate-buffered saline.

Figure 3. Effects of atrogin-1 siRNA on the levels of autophagy-related proteins in C2C12 myotubes. Data are expressed as the mean ± SEM in each group (n = 6). The two-tailed *t*-test was used to analyze the differences between the groups. **,*,Significance with respect to the NT group ($P < 0.01$ and $P < 0.05$, respectively). NT, non-targeting siRNA; siAtr, atrogin-1 siRNA.

Fig 1

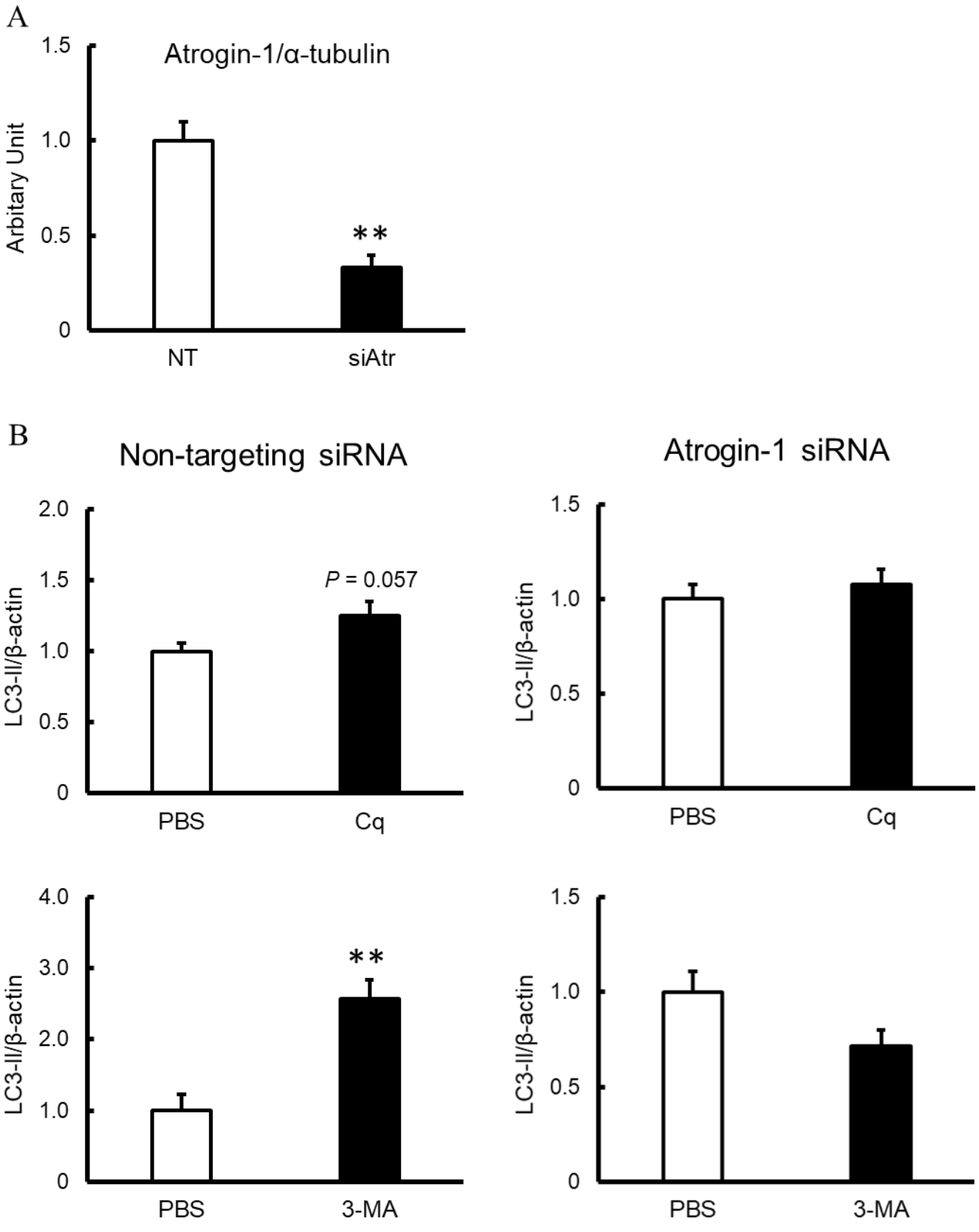
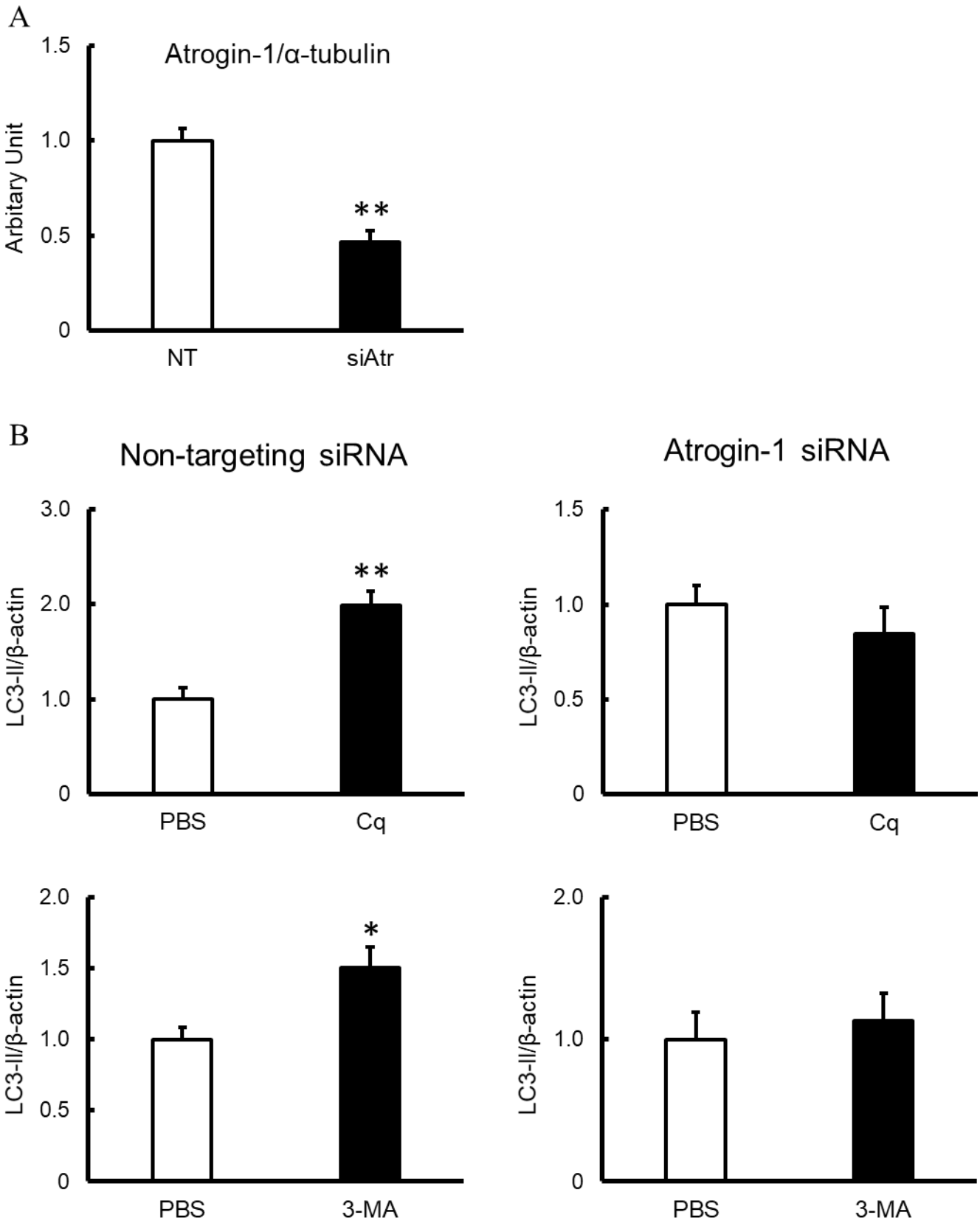

A
Atrogin-1/α-tubulin
Arbitary Unit
1.5
1.0
0.5
0
**
NT
siAtr
B
Non-targeting siRNA
Atrogin-1 siRNA
LC3-II/β-actin
2.0
1.5
1.0
0.5
0
P = 0.057
PBS
Cq
4.0
3.0
2.0
**
3-MA

Fig 2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Atrogin-1/α-tubulin
Arbitary Unit
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NT
siAtr
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Non-targeting siRNA
Atrogin-1 siRNA
LC3-II/β-actin
3.0
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PBS
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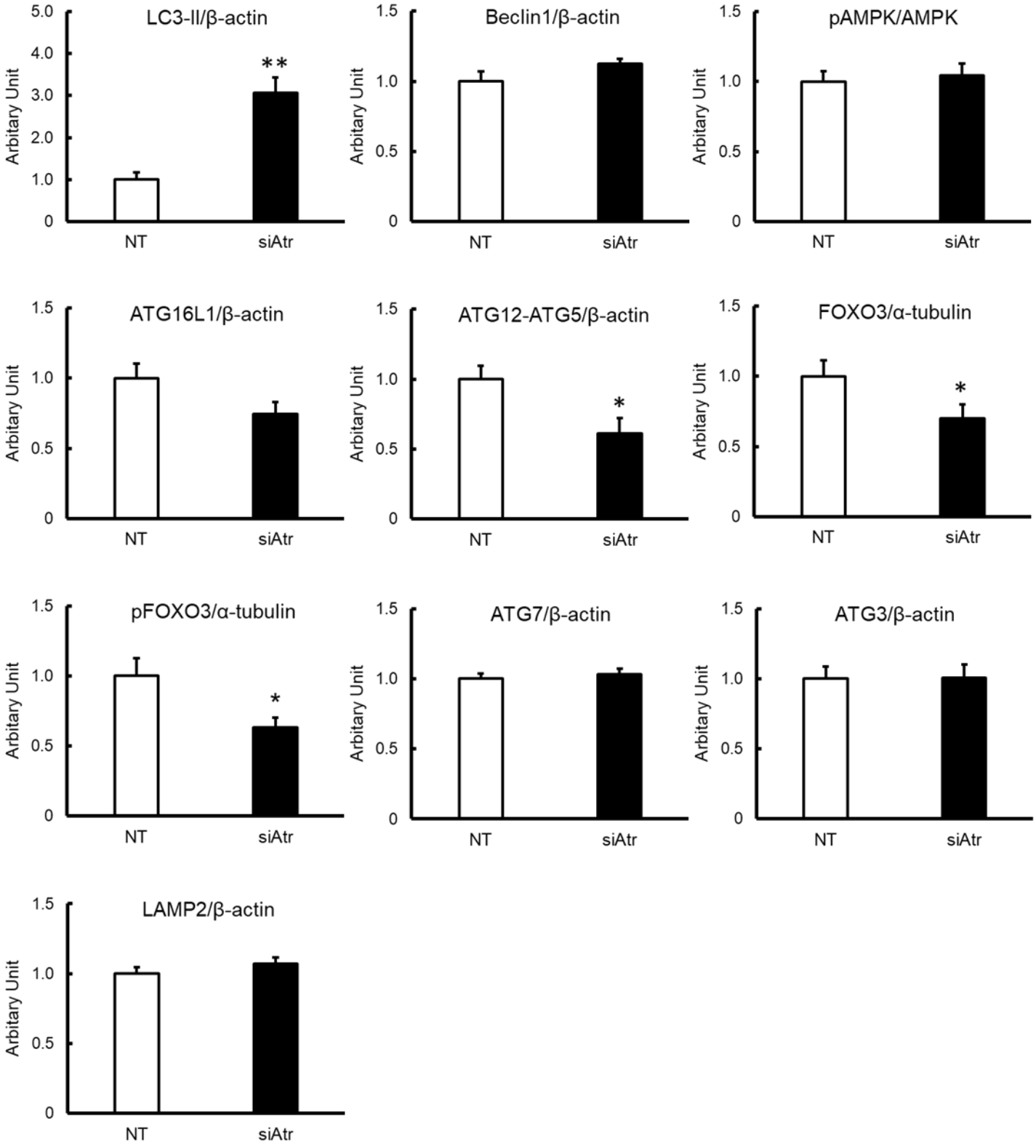
Fig 3


LC3-II/β-actin
Beclin1/β-actin
pAMPK/AMPK
ATG16L1/β-actin
ATG12-ATG5/β-actin
FOXO3/α-tubulin
pFOXO3/α-tubulin
ATG7/β-actin
ATG3/β-actin
LAMP2/β-actin
Arbitary Unit
NT
siAtr
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*