〈Original Article〉

Effect of renalase on dexamethasone-induced muscle atrophy

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Summary Muscle atrophy can be induced by dexamethasone, a glucocorticoid. This model enhanced the muscle protein degradation pathway. Renalase is a flavin adenine dinucleotide-dependent amine oxidase that was discovered in 2005 and expressed mainly in the kidney. Renalase has an inhibitory effect on apoptosis and inflammation, thereby promoting cell survival. Renalase in skeletal muscles restrained a muscle ring finger protein (MuRF-1) expression via phosphorylation of protein kinase B (Akt) after acute exercise. However, whether renalase is related to muscle atrophy is unclear. This study aimed to elucidate the renalase expression related to the dexamethasone-induced muscle atrophy model *in vitro*. The C2C12 myotubes were treated with either dimethyl sulfoxide (DMSO) or dexamethasone (DEX) under various conditions. The renalase protein and mRNA expression levels were highest in the 10 μmol/L DEX group for 24 h under various DEX conditions. In addition, the renalase, MuRF-1, and muscle atrophy F-box mRNA expression levels were higher at 48 h than in other time courses. Moreover, the renalase protein expression level was observed to increase on treatment with 10 μmol/L DEX for 48 h, although the myotube width and cell survival ratio decreased ($p < 0.01$). Notably, the mRNA expression levels of REDD 1 and KLF 15 were increased and the phosphorylation of Akt was decreased by the condition ($p < 0.01$). Additionally, the phosphorylation of Akt in 48 h recovered compared with that in 24 h. In conclusion, the results suggest that the renalase expression restrain dexamethasone-induced muscle atrophy via Akt pathway.

Key words: Akt, MuRF-1, MAFbx

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1. Introduction

The skeletal muscle comprises approximately 40% of the body mass in humans. Muscle is a plastic tissue that can undergo hypertrophy and/or atrophy. Muscle atrophy may be caused by aging, malnutrition, steroids, and physical inactivity. In previous studies, muscle atrophy was induced by dexamethasone[1-3]. Dexamethasone-induced muscle atrophy increased the ubiquitin proteasome pathway, including muscle ring finger protein (MuRF-1) and muscle atrophy F-box (MAFbx)[4,5]. In addition, kruppel-like factor 15 (KLF 15), which works in the ubiquitin proteasome pathway, and regulated in development and DNA damage responses 1 (REDD 1), which restrains the protein synthesis-dependent mechanistic target of rapamycin (mTOR), are identified as a target of the glucocorticoid receptor (GR)[6,7].

Renalase is a flavin adenine dinucleotide-dependent amine oxidase[8], and its primary function is to metabolize catecholamines and regulate blood pressure[8,9]. Although renalase is expressed primarily in the kidney, its expression has also been identified in other tissues, including skeletal muscles, cardiomyocytes, and the liver[8]. Previous studies suggested that renalase also has an inhibitory effect on apoptosis and inflammation, thereby promoting cell survival[10-13]. Renalase exerts its protective effects by phosphorylating protein kinase B (Akt) via the renalase receptor plasma membrane Ca^{2+} ATPase isoform 4 b (PMCA 4b)[14,15]. A previous study showed that during skeletal muscle exercise, renalase reduced the expression level of MuRF-1, a gene related to muscle protein degradation, via phosphorylation of Akt[16].

We hypothesized that skeletal muscle atrophy would be related to renalase expression. This study was aimed at clarifying the relationship between dexamethasone-induced muscle atrophy and renalase expression.

2. Materials and Methods

Cell culture

C2C12 myoblast cells (RCB0987, Lot#: 37, RIKEN BRC, Japan) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Wako Pure Chemical Industries, Ltd., Japan) containing 10% fetal bovine serum, 1% penicillin, and streptomycin. After the cultures reached 70–80% confluence, the medium was replaced with DMEM containing 2% horse serum (differentiation medium) and changed every 2 days.

Dexamethasone-induced muscle atrophy

On the 5th day, the myotubes were treated with either dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., USA) or dexamethasone (DEX; Fujifilm Co., Japan). After incubation under various conditions, samples were collected to extract protein and mRNA, and stored at −80 °C for subsequent analyses.

Quantitative real-time RT-PCR

Total mRNA was isolated using the Sepasol®-RNA Super G kit (Nacalai, Japan) according to the manufacturer's instructions. Reverse transcription was performed using thermal cycler (Thermal cycler Dice® Touch, TAKARA BIO INC., Japan) added to PrimeScript™ RT Master Mix (Perfect Real Time; TAKARA BIO INC., Japan) after setting up the same concentration in total RNA. The mRNA expression in each target was carried out using a KAPA SYBR FAST qPCR kit (Kapa Biosystems, USA) and the real-time PCR system (Quant Studio 5, Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal normalizing control for the mRNA. The primer sequences used in this study are shown in Table 1.

Western blot analysis

The proteins (each 10 μg/lane) were resolved by SDS-PAGE and transferred to a polyvinylidene

Table 1 Primer sequences used in the mRNA analyses

Name	Forward (5' -> 3')	Reverse (5' -> 3')
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Myog	CCTTGCTCAGCTCCCTCA	TGGGAGTTGCATTCACTGG
Myh 4	TGCCTCCTTCTTCATCTGGT	CCATCTCAGCGTCGGAAC
Renalase	TGACCTTGTCATCCTCACCA	TCCCTCTGGCGTTCACTAAT
Trim 63	TGATTCCTGATGGAAACGCTATGG	ATTCGCAGCCTGGAAGATGTC
Fbxo 32	GACAAAGGGCAGCTGGATTGG	TCAGTGCCCTTCCAGGAGAGA
Ddit 4	CCAGAGAAGAGGGCCTTGA	CCATCCAGGTATGAGGAGTCTT
Klf 15	CGGTGCCTTGACAACTCATC	AAATGCACTTTCCCAGGCTG

Gapdh: glyceraldehyde-3-phosphate dehydrogenase
Myog: Myogenin
Myh 4: Myocine heavy chain 4
Trim63 (MuRF-1): tripartite motif containing 63(Muscle RING finger protein)
Fbxo32 (MAFbx): F-box protein 32 (Muscle atrophy F-box)
Ddit 4 (REDD 1): DNA-damage-inducible transcript 4 (regulated in DNA damage and development 1)
Klf 15: Krüppel-like factor 15

fluoride membrane (GE Healthcare Life science, Germany). The PVDF membranes were blocked with 5% skim milk in TBS-T (0.1% Tween 20). Subsequently, they were incubated with the primary antibodies overnight at 4°C while shaking. Following washes, the membranes were incubated with HRP-conjugated secondary antibodies for 60 min at room temperature. Post washing, the membranes were treated with a chemiluminescent reagent (ECL Select Western Blotting Detection Reagent, GE Healthcare, USA) and imaged using ImageQuant LAS-4000 (GE Healthcare Life science, Japan). The signals were analyzed using Image J. The signals of renalase (Abcam, ab178700), Akt (9272, Cell Signaling Technology), p-Akt (13038, Cell Signaling Technology), β-actin (SANTA CRUZ, sc-81178), and β-tubulin (SANTA CRUZ, sc-5274) were measured. Notably, β-actin or β-tubulin was analyzed as an internal standardization control.

Observation and cell counting

C2C12 myotubes were observed under a microscope (BZ-X710, Keyence, Japan), and images were taken. Five myotube cells were selected randomly from each well (6-well plate) in accordance with previous studies[3,5]. Myotube widths measured from these images were analyzed using Image J. Cell count was performed using Cell Counting Kit 8 (Dojin, Japan) in accordance with the manufacturer's instructions.

Statistical analysis

Data are shown as mean ± SD. For all measurements, a one-way analysis of variance was used to evaluate significance. Statistical analyses were conducted using the GraphPad Prism 7 software (GraphPad, Inc., La Jolla, CA, USA). The data were subjected to t-tests for the comparison between the two groups; P values below 0.05 were considered significant.

3. Results

Differentiation of C2C12 myotube

Confirmation of myotubes showed greater myogenin and myosin heavy chain expression levels on day 5 of the C2C12 differentiation than the previous differentiation (Fig. 1).

Time course and concentration on DEX

The renalase protein expression was

significantly increased by 10 μmol/L DEX upon incubation for 24 h with DEX as compared to that with DMSO. In addition, the mRNA expression of renalase and atrogenes such as MuRF-1 and MAFbx were increased by the condition. On the other hand, phosphorylation of Akt was decreased by the condition (Fig. 2).

Forty-eight hours was the optimal time course as the mRNA expression of renalase and atrogenes (MuRF-1 and MAFbx) was significantly increased by DEX (Fig. 3).

Renalase expression, cell death, and width on the condition

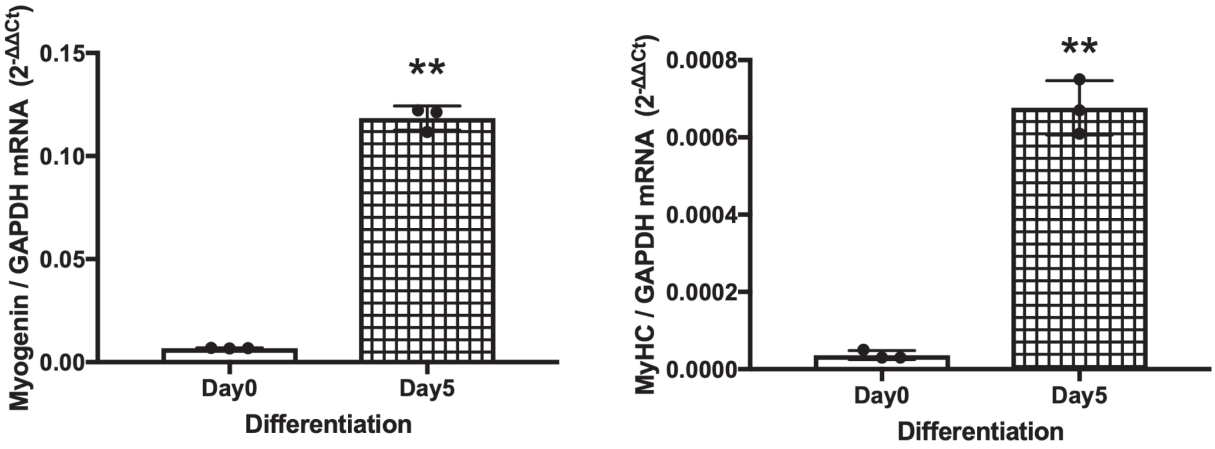

Fig. 1 Differentiation markers of the C2C12 cells
C2C12 myoblasts differentiated into myotubes by 2% horse serum medium. The asterisk shows a significant difference as compared with Day 0 (**p < 0.01). Data are shown as mean ± SD. n = 3 in each group.

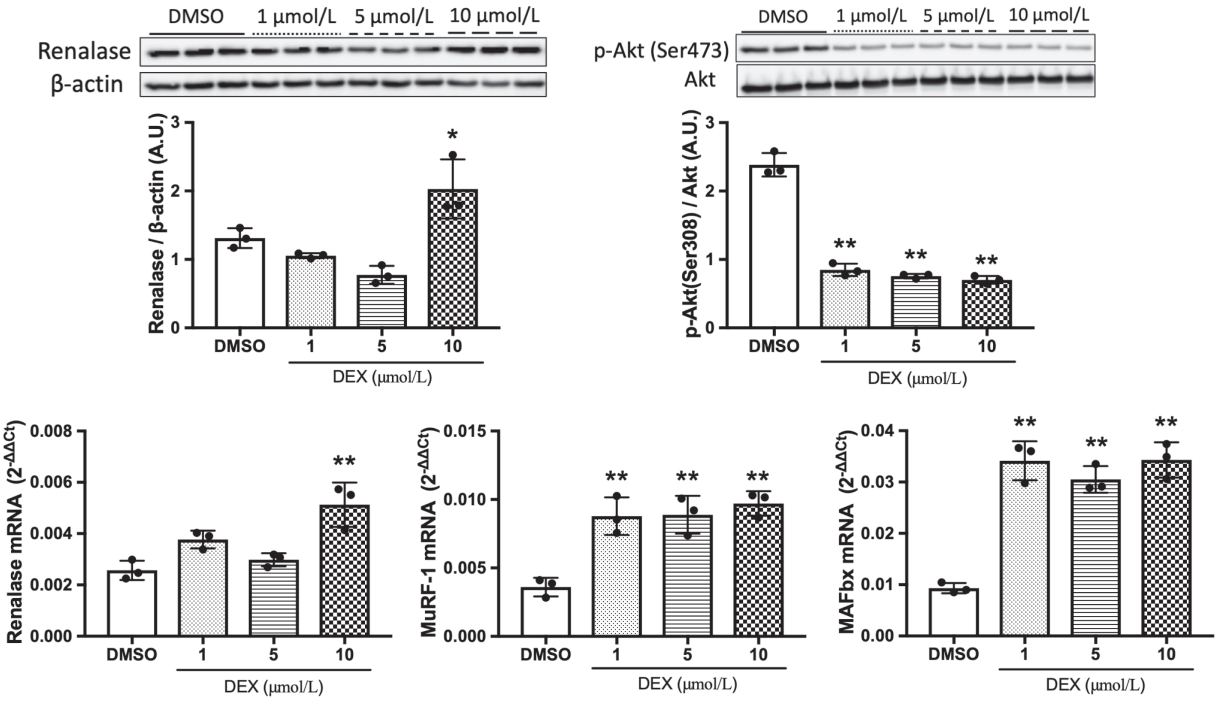

Fig. 2 Renalase, Akt, MuRF-1, and MAFbx expression levels were increased by various concentrations for 24 h
C2C12 myotubes were stimulated by various concentrations of DEX for 24 h. The asterisk shows a significant difference as compared with DMSO (*p < 0.05, **p < 0.01). Data are shown as mean ± SD. n = 3 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone.

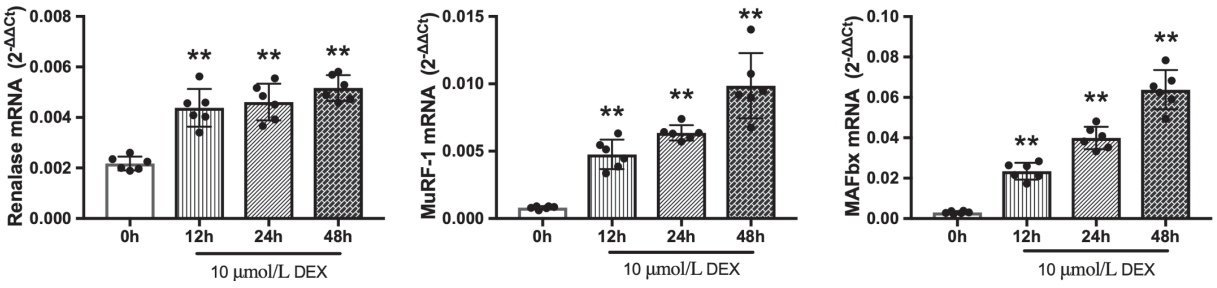

Fig. 3 The mRNA expression levels of renalase and atrogenes were increased at various time courses with 10 μmol/L DEX

C2C12 myotubes were stimulated at various time courses with 10 μmol/L DEX. The asterisk shows a significant difference as compared with 0 h incubation (**p < 0.01). Data are shown as mean ± SD. n = 6 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone.

The renalase protein expression was elevated by treatment with 10 μmol/L DEX for 48 h although the myotube width and cell survival ratio were lowered (Fig. 4). The mRNA expression of REDD 1 and KLF 15 was increased, and phosphorylation of Akt was decreased by the condition (Fig. 4).

4. Discussion

The purpose of this study was to investigate the effect of renalase expression on dexamethasone-induced muscle atrophy. We determined the model because it is the most common model for muscle atrophy *in vitro*. First, we investigated the differentiation in C2C12 cells. As a result, the C2C12 myotube was of a higher value than myoblasts based on the differentiation factors (Fig. 1). Therefore, we conducted the subsequent experiments on the conditions of differentiation.

The mRNA expression levels of MuRF-1 and MAFbx increased in all concentrations for 24 h. In addition, the phosphorylation of Akt was decreased by the condition. These results were in agreement with those in previous studies[1-5]. Moreover, the expression levels of renalase both protein and mRNA were increased by the condition. C2C12 was stimulated by 10 μmol/L DEX for some time course. Notably, 48 h was the optimal time course because the mRNA expression levels of renalase and atrogenes (MuRF-1 and MAFbx) were significantly increased by DEX, although all the time courses showed increased mRNA expression levels of renalase and atrogenes. From the results obtained, the condition stimulated by 10 μmol/L DEX for 48 h was determined to be the most optimal for conducting the following experiment.

When C2C12 myotube was stimulated by 10 μmol/L DEX for 48 h, the myotube width and cell counting were decreased. Thus, C2C12 myotube caused dexamethasone-induced muscle atrophy. In addition, the expression levels of REDD 1 and KLF 15, which restrain the mTOR and regulate atrogenes and mRNA expressions, respectively, were increased by the condition. Therefore, the experiment supported previous studies and was affected by the GR[6,17]. The renalase protein expression level was significantly increased by the condition. Tokinoya et al. (2018) suggested that increases in the expression of renalase in the skeletal muscles are related to protein degradation via the Akt pathway[16]. The phosphorylation of Akt was decreased by DEX, as the signaling was decreased by DEX in previous studies[2,5]. This is the reason why upstream factors of Akt were restrained directly by dexamethasone in C2C12[18]. In addition, myostatin, which was increased by DEX[19], decreased the phosphorylation of Akt. Although the renalase expression level was increased by dexamethasone-induced muscle atrophy, the effect on atrogenes is still unknown. Our previous study suggested that the renalase expression level can reduce the MuRF-1 expression level during acute exercise[16]. Acute exercise

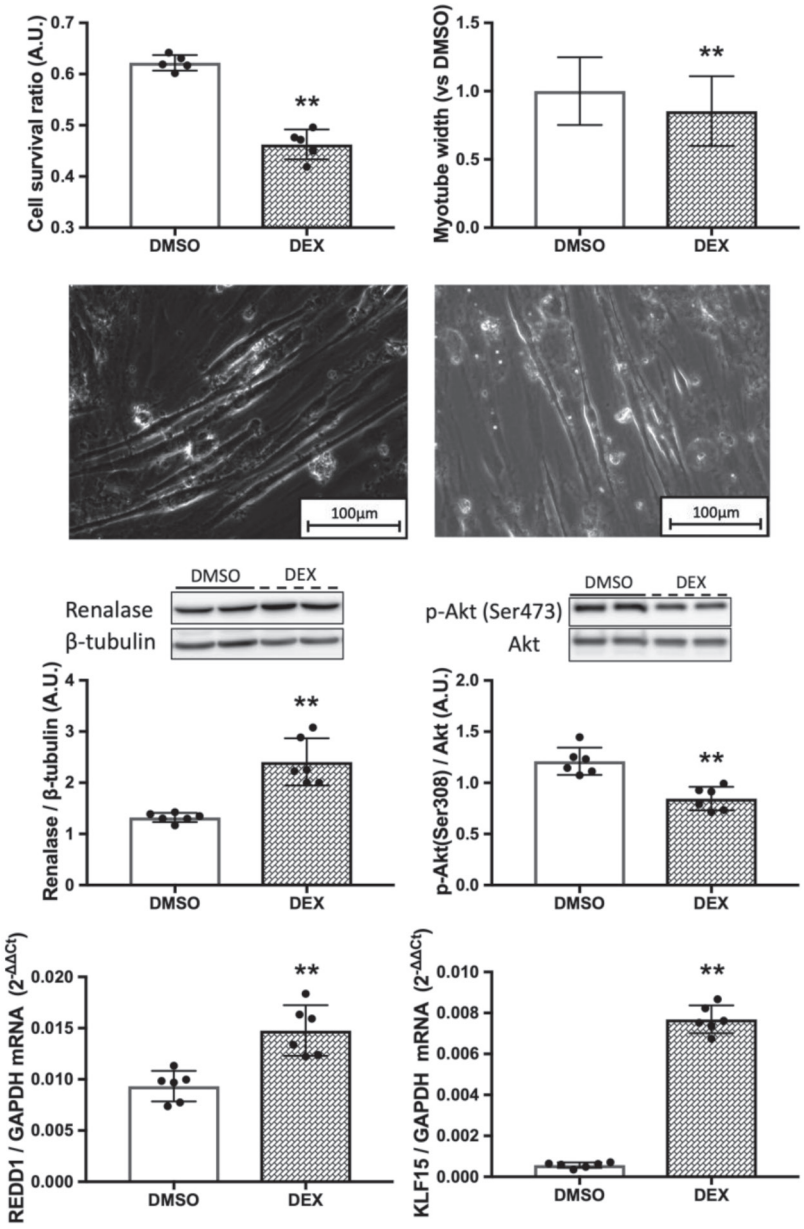

Fig. 4 Changes in renalase expression and muscle atrophy factors by 10 μmol/L DEX for 48 h

C2C12 myotubes were stimulated by 10 μmol/L DEX for 48 h. Myotube widths were quantified in 30 randomly selected fields. The asterisk shows a significant difference as compared with DMSO (**$p < 0.01$). Data are shown as mean ± SD. n = 6 in each group. DMSO, dimethyl sulfoxide; DEX, dexamethasone.

produced reactive oxygen sepsis (ROS), and Akt is known to be a potent activator of ROS[18]. Therefore, it might be a problem of the signaling related to Akt because many factors activate the phosphorylation of Akt in an acute exercise model, whereas the DEX model in this study inhibited the phosphorylation of Akt by various factors. However, renalase induces the phosphorylation of Akt to protect cells from acute kidney disease[15]. Our results showed that the phosphorylation of Akt in 48 h recovered compared with that in 24 h because it might be the result of renalase activating the Akt expression via the

receptor. In future studies, the verification of renalase overexpression or knock down on dexamethasone-induced muscle atrophy must be clarified. In conclusion, this study suggests that the renalase expression level restrain dexamethasone-induced muscle atrophy. In the further study, we need to research overexpression and knock out of renalase on muscle atrophy.

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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