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Morin attenuates dexamethasone-mediated oxidative stress and atrophy in mouse C2C12 skeletal myotubes

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ABSTRACT

Glucocorticoids are the drugs most commonly used to manage inflammatory diseases. However, they are prone to inducing muscle atrophy by increasing muscle proteolysis and decreasing protein synthesis. Various studies have demonstrated that antioxidants can mitigate glucocorticoid-induced skeletal muscle atrophy. Here, we investigated the effect of a potent antioxidative natural flavonoid, morin, on the muscle atrophy and oxidative stress induced by dexamethasone (Dex) using mouse C2C12 skeletal myotubes. Dex (10 μM) enhanced the production of reactive oxygen species (ROS) in C2C12 myotubes via glucocorticoid receptor. Moreover, Dex administration reduced the diameter and expression levels of the myosin heavy chain protein in C2C12 myotubes, together with the upregulation of muscle atrophy-associated ubiquitin ligases, such as muscle atrophy F-box protein 1/atrogin-1, muscle ring finger protein-1, and casitas B-lineage lymphoma proto-oncogene-b. Dex also significantly decreased phosphorylated Foxo3a and increased total Foxo3a expression. Interestingly, Dex-induced ROS accumulation and Foxo3a expression were inhibited by morin (10 μM) pretreatment. Morin also prevented the Dex-induced reduction of myotube thickness, together with muscle protein degradation and suppression of the upregulation of atrophy-associated ubiquitin ligases. In conclusion, our results suggest that morin effectively prevents glucocorticoid-induced muscle atrophy by reducing oxidative stress.

1. Introduction

Synthetic glucocorticoids are used to manage various pathological abnormalities linked to inflammation. Despite their beneficial effects, a high dose or sustained use of these drugs cause serious side effects, including muscle atrophy. Dexamethasone (Dex) is a long-acting synthetic glucocorticoid that instigates skeletal muscle atrophy by stimulating muscle proteolysis and suppressing protein synthesis [1]. Dex can induce muscle atrophy via two pathways, i.e., the GR-mediated gene expression pathway and the oxidative-stress-mediated pathway [2–5].

In glucocorticoid receptor (GR)-mediated gene expression pathway, Dex interacts with GR and induces the expression of Krüppel-like factor 15 (KLF15), a member of the zinc finger transcription factor family of proteins. KLF15 acts as a catabolic modulator of skeletal muscle via the direct transcriptional upregulation of the ubiquitin ligases muscle atrophy F-box protein 1 (MAFbx1)/atrogin-1 and muscle ring finger protein-1 (MuRF-1), which are associated with muscle atrophy [2]. Conversely, muscle atrophy is closely associated with oxidative stress

Abbreviations: Cbl-b, casitas B-lineage lymphoma proto-oncogene-b; Dex, dexamethasone; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FoxO, forkhead box O; GPx1, glutathione peroxidase; GR, glucocorticoid receptor; H2-DCFDA, 2',7'-dicholorofluorescein diacetate; IGF-1, insulin like growth factor 1; IRS-1, insulin receptor substrate-1; KLF15, Krüppel like factor 15; MAFbx1, muscle atrophy F-box protein 1; MuRF-1, muscle ring finger protein-1; MyHC, myosin heavy chain; NT, non-targeting; PGC-1α, peroxisome proliferator-activated receptor-gamma co-activator 1-alpha; ROS, reactive oxygen species; SOD1, superoxide dismutase-1; TEAC, Trolox equivalent antioxidant capacity.

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[6], which is an imbalance between pro-oxidants and antioxidants that promotes protein catabolism and depresses protein synthesis [7]. Dex generates oxidative stress in skeletal muscle cells *in vitro* and *in vivo* [3,5,8], as well as in other type of cells, such as adipocytes, osteoblastic cells, and pancreatic cells [9,10]. We reported previously that the accumulation of ROS in rodent myotubes under real and simulated microgravity conditions significantly upregulated the ubiquitin ligase casitas B-lineage lymphoma proto-oncogene-b (Cbl-b), which induced muscle atrophy through the stimulation of the degradation of insulin receptor substate-1 (IRS-1) [11]. Disturbed insulin-like growth factor-1 (IGF-1) signaling also stimulated the expression of MAFbx1/atrogin-1 and MuRF-1 [12]. Thus, oxidative stress may be a suitable target to inhibit the Dex-mediated skeletal muscle atrophy.

Polyphenols are natural compounds that are present in vegetables and have a high antioxidant activity, low toxicity, and the structural flexibility to interact with nucleic acids and enzymes [13,14]. Many studies have demonstrated that polyphenols can effectively attenuate Dex-induced muscle atrophy by downregulating muscle-atrophy-related proteins, such as the ubiquitin ligases MAFbx1/atrogin-1 and MuRF-1 [15–17]. We previously found that dietary quercetin prevented disuse muscle atrophy by targeting mitochondria in denervated mice [18]. More recently, quercetin was also shown to be protective against Dex-induced muscle injury by regulating apoptosis [8]. Morin, a yellowish pigmented bioflavonoid and structural isomer of quercetin, is abundantly found in members of the *Moraceae* family. However, the information on the effects of morin on skeletal muscle is scarce. To evaluate the effects of morin on skeletal muscle atrophy, we treated C2C12 myotubes with Dex in the presence or absence of morin and measured ROS production, muscle atrophy-associated molecules, and myotube diameter.

2. Materials and Methods

2.1. Chemicals

Morin, quercetin, dexamethasone (Dex), and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO). Piceatannol and resveratrol were purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.2. Measurement of antioxidant capacity

The antioxidant capacity of phenolic compounds morin, quercetin, piceatannol, and resveratrol was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a substrate and an antioxidant assay kit (Dojindo Laboratories, Kumamto, Japan) [19]. Briefly, the optimum concentration range of samples encompassing 50% scavenging was determined. Subsequently, the IC_{50} was calculated by replotting the regression line based on the obtained range of sample concentrations for 50% scavenging. The results of this analysis are expressed as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) equivalent antioxidant capacity (TEAC).

2.3. Cell culture

Mouse C2C12 myoblasts were obtained from the American Type Culture Collection and maintained and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma–Aldrich) containing 10% fetal bovine serum (Thermo Fischer Scientific, Waltham, MA) and a 1% penicillin−streptomycin mixed solution (Nacalai Tesque, Kyoto, Japan) at 37 °C in 5% CO_2 humidified air until reaching 100% confluency; thereafter, the culture medium was replaced with DMEM containing 2% horse serum (Thermo Fischer Scientific), for differentiation into myotubes. All media were changed at 48 h intervals. After the myotubes were fully differentiated, they were treated with 10 μM morin for 1 h as a pretreatment, followed by treatment with 10 μM dexamethasone (Dex) for 24 h to induce muscle fiber atrophy. DMSO (0.01%) and Milli-Q water were used as the vehicle for morin and Dex, respectively. The dose of morin (10 μM) was selected based on our random study of three independent doses (10, 50, and 100 μM) of morin and their effectiveness (data not shown). Finally, cells were harvested, processed, and subjected to qRT−PCR and Western blot analysis.

2.4. Measurement of myotube diameter

To analyze the diameter of C2C12 myotubes, 10 images per group of myotube culture were captured using a phase-contrast microscope (BIOREVO, BZ-9000; Keyence, Osaka, Japan) at 20 × magnification. One hundred myotubes/group were measured on a random basis from 10 micrographs. The thickest portion of each myotube was chosen for diameter measurement using the BZ-II analyzer software (Keyence).

2.5. Cytotoxicity assay

For the cytotoxicity assay, C2C12 cells were seeded at 0.05×10^5 cells per well in 96 well plate, cultured and differentiated to myotubes as shown in sub-section 2.3. Myotubes were treated with 10 μM Dex for 24 h in the presence or absence of 10 μM morin as 1 h pretreatment. After the treatment, LDH test was performed using Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer's protocol.

2.6. Quantitative real time (RT)−PCR

Real-time polymerase chain reaction (RT−PCR) was performed using the Power SYBER Green PCR Master Mix™ (Thermo Fischer Scientific) on a StepOnePlus™ Real-Time PCR system (Thermo Fischer Scientific). Briefly, total RNA was collected from cells using ISOGEN™ (Nippon Gene, Tokyo, Japan). The isolated RNA was quantified on a Nanodrop 1000 Spectrophotometer (Thermo Fischer Scientific), followed by reverse transcription to cDNA. The sequences of the primers used in this experiment are shown in Table 1. The 18S ribosomal RNA was used as an internal standard.

2.7. Western blotting

Lysis buffer containing 50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 5

Table 1
Primers used for PCR.

Target gene		Sequence	Length (bp)
MAFbx1/atrogin-1	S	GGCGGACGGCTGGAA	101
	AS	CAGATTCTCCTTACTGTATACCTCCTTGT	
MuRF-1	S	TGTCTGGAGGTCGTTTCCG	183
	AS	CTCGTCTTCGTGTTCCTTGC	
Cbl-b	S	GAGCCTCGCAGGACTATGAC	222
	AS	CTGGCCACTTCCACGTTATT	
SOD1	S	ACCAGTGCAGGACCTCATTTTAA	78
	AS	TCTCCAACATGCCTCTCTTCATC	
Catalase	S	ATGGCTTTTGACCCAAGCAA	69
	AS	CGGCCCTGAAGCTTTTTGT	
GPx1	S	GCGGCCCTGGCATTG	118
	AS	GGACCAGCGCCCATCTG	
KLF-15	S	CCAGGCTGCAGCAAGATGTACAC	125
	AS	TGCCTTGACAACTCATCTGAGCGG	
18sr	S	CATTCGAACGTCTGCCCTA	119
	AS	CCTGCTGCCTTCCTTGGA	
GR	S	CAAAGCCGTTTCACTGTCC	296
	AS	ACAATTTCACACTGCCAC	

18Sr, 18S ribosomal RNA; Cbl-b, Casitas B-lineage lymphoma proto-oncogene-b; GPx1, glutathione peroxidase 1; GR, glucocorticoid receptor; KLF15, Krüppel-like factor 15; MuRF-1, muscle ring finger protein-1; SOD1, superoxide dismutase 1.

mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 1% Triton-X-100, a protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland), and 10 μM MG-132 was used. Protein concentration in the cell homogenates was quantified using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific) and bovin serum albumin (BSA) as standard following manufacturer's protocol. Ten microgram of protein per lane were electrophoresed on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis at 300 V for approximately 1 h, followed by transfer to a polyvinylidene difluoride membrane. The membrane was then blocked with Milli-Q water containing 4% block ACE™ powder (DS Pharma Biomedical Co. Ltd., Osaka, Japan) for 1 h. Respective incubation with the primary antibody and secondary antibody was performed overnight at 4 °C and 1 h at room temperature. The blots were subjected to densitometry using a C-DiGit scanner (LI-COR Biosciences, Lincoln, NE). Antibodies against fast- or slow-type myosin heavy chain (MyHC), alpha-tubulin (Sigma), Foxo3a, phosphorylated-Foxo3a, and rabbit IgG (Cell Signaling Technology, Danvers, MA), P66shc and peroxisome proliferator activator receptor γ coactivator-1α (PGC-1α) (Abcam, Cambridge, UK) and glucocorticoid receptor (GR) (Santa Cruz Biotechnology, Dallas, Texas) were used in this analysis.

2.8. Measurement of reactive oxygen species

Myotubes grown on 96-well plates were washed with medium and incubated with 5 μM H2-DCFDA reagent (Thermo Fischer Scientific) in medium for 1 h at 37 °C. After incubation, myotubes were washed with Hank's Balanced Salt Solution (HBSS) twice and fluorescence was determined using a microplate reader (INFINITE M NANO™; Tecan, Mannedorf, Switzerland) at an excitation wavelength of 495 nm and an emission wavelength of 527 nm according to manufacturer's protocol.

2.9. Small interfering RNA and transfections

We used siRNA technique for specific knockdown of GR mRNA expression. C2C12 myotubes were transfected with the GR siRNA or non-targeting (NT) siRNA (Thermo Fischer Scientific) using the transfection reagent lipofectamine RNAiMAX (Thermo Fisher Scientific). The siRNA used for mouse GR knockdown was 5′- GCAUGUAUGACCAAUGUAAtt-3'. The siRNA for NT from the supplier (Thermo Fischer Scientific) was used as negative control but the sequence was not opened. The siRNA construct was used at a final concentration of 25 nM with medium in combination with lipofectamine, according to the manufacturer's instructions. Twenty-four hours later the medium was replaced with fresh medium, and myotubes were treated with Dex (10 μM) and/or morin as mentioned in subsection 2.3.

2.10. Statistical analysis

Unpaired Student's t-test was used to compare two samples. One-way ANOVA and Scheffe's test were used for the comparison of more than two samples. The results are presented as means ± SD. Significance was set at $P < 0.05$.

3. Results

3.1. Antioxidant capacity of phenolic compounds

The antioxidant capacity of the tested polyphenols was estimated based on the extent of the reduction of DPPH. The IC_{50} and TEAC values of the tested polyphenols are shown in Table 2. Among the tested polyphenols, quercetin was the most potent antioxidant, whereas resveratrol showed the lowest scavenging activity. Morin, an analog of quercetin, exhibited a higher scavenging effect than did resveratrol, and a similar efficacy to that of piceatannol.

Table 2
Antioxidant capacity of the different polyphenolic compounds.

Sample	IC_{50} (μM)	TEAC (μM)
Quercetin	83.6	3.01
Morin	182	1.38
Piceatannol	172	1.46
Resveratrol	687	0.36

The DPPH assay method was used for the measurement of antioxidant capacity. The values of IC_{50} and trolox equivalent antioxidant capacity (TEAC) were calculated using the following formulas: inhibition ratio of sample (%) = (absorbance of the blank− absorbance of the sample)/absorbance of the blank × 100; and TEAC = IC_{50} of Trolox/IC_{50} of the sample.

3.2. Effect of morin on Dex-mediated myotube atrophy and muscle protein degradation

Dex significantly reduced C2C12 myotube thickness compared with vehicle-treated control myotubes (Fig. 1A). In turn, morin pretreatment effectively attenuated the Dex-induced reduction of the thickness of C2C12 myotubes. Morin administration alone retained the diameter of myotubes comparable to the control group.

The LDH test was performed to detect the cytotoxicity of Dex and morin on C2C12 myotubes. Both Dex and/or morin had no effect on release of LDH (Fig. 1A), indicating that both compounds exerted no cytotoxicity on C2C12 myotubes.

Next, we investigated the effects of Dex and/or morin on the fast- and slow-type MyHC proteins in C2C12 myotubes using Western blot analysis. Compared with the vehicle-treated control, fast-type MyHC was significantly decreased in C2C12 myotubes treated with 10 μM Dex for 24 h (Fig. 1B). Morin prevented the downregulation of fast-type MyHC induced by Dex, whereas morin treatment alone tended to decrease the levels of fast-type MyHC compared with the control. In contrast, Dex treatment did not affect slow-type MyHC significantly compared with vehicle-treated C2C12 myotubes. Interestingly, morin significantly increased the levels of slow-type MyHC, regardless of Dex treatment (Fig. 1B). These results suggest that morin is an effective agent for the suppression of Dex-induced muscle atrophy.

We also examined the effect of morin on the expression of muscle-atrophy-associated ubiquitin ligases in response to Dex administration. Treatment with 10 μM Dex for 24 h significantly increased the expression of all tested ubiquitin ligases compared with the vehicle treatment **(Fig. 1C)**. Morin pretreatment significantly attenuated the Dex-induced upregulation of these ubiquitin ligases in C2C12 myotubes, whereas morin treatment alone had no effect on the expression of these proteins. Meanwhile, morin failed to suppress the Dex-induced KLF-15 upregulation **(Fig. 1C)**. These findings indicate that morin has a lesser effect on the canonical GR-mediated pathway associated with the down-regulation of muscle atrophy-associated ubiquitin ligases.

3.3. Suppressive effect of morin on Dex-induced oxidative stress in C2C12 myotubes

To elucidate whether morin can prevent myotube atrophy as an antioxidant, we evaluated the effect of morin on Dex-induced ROS levels. A significant increase in ROS levels in C2C12 myotubes was observed after Dex treatment compared with vehicle-treated control myotubes (Fig. 2A). Morin pretreatment significantly suppressed the Dex-induced ROS accumulation in C2C12 myotubes, although treatment with morin alone did not affect ROS levels. Dex significantly increased the level of P66shc, an oxidative stress marker [20] compared with vehicle treatment (Fig. 2B). As expected, morin significantly suppressed the Dex-induced upregulation of P66shc. The expression of the antioxidant enzymes, such as superoxide dismutase (SOD1), catalase, and glutathione peroxidase (GPx1), was also determined (Fig. 2C). Dex significantly suppressed the expression of SOD1 and catalase compared

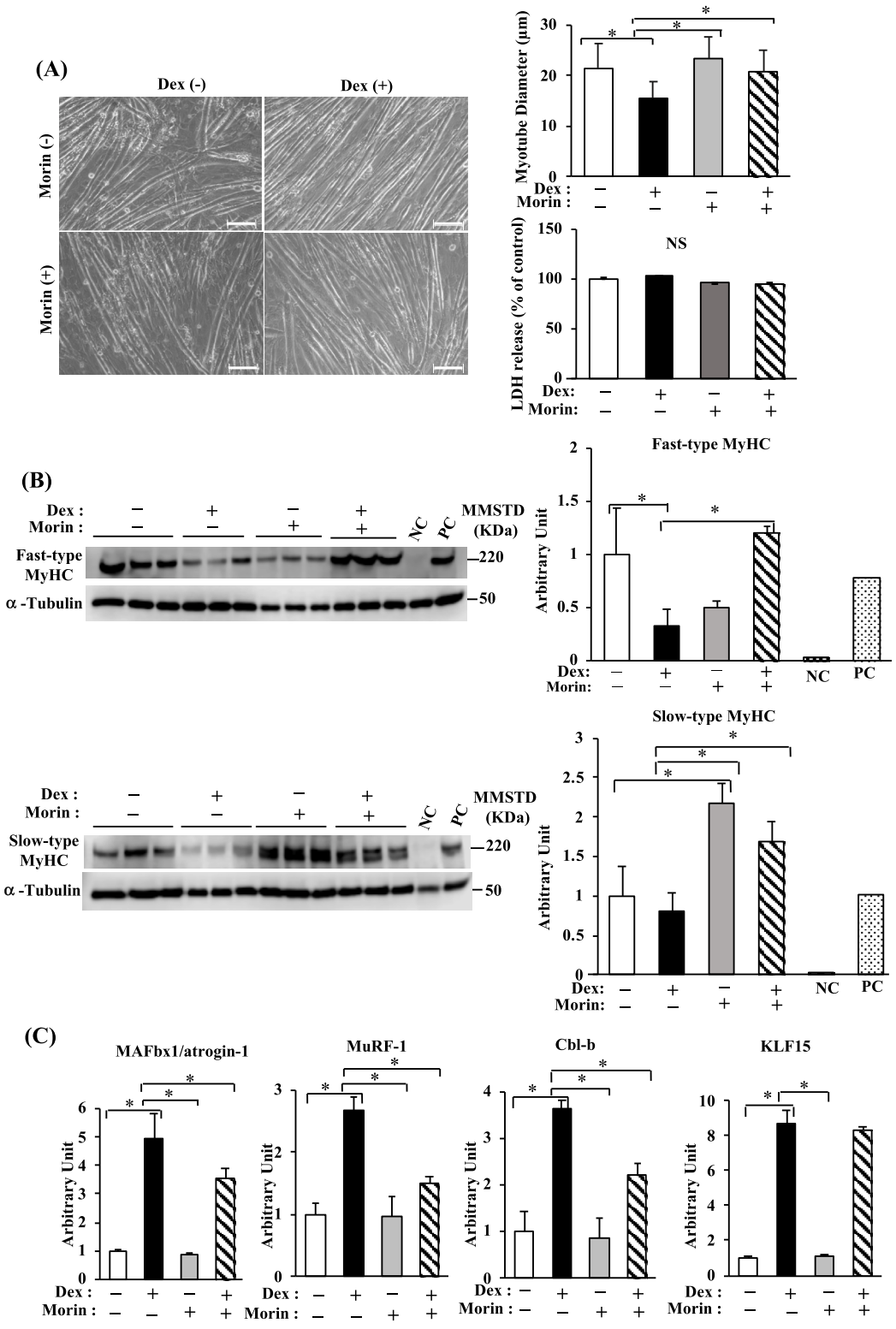

Fig. 1. Morin prevented the Dex-induced atrophy of C2C12 myotubes. Myotubes were treated with 10 μM dexamethasone for 24 h with or without 10 μM morin as a pretreatment for 1 h. **(A)** Myotube morphology and diameter. The diameter was measured as mentioned in section 2.4. The results are expressed as means ± SD (n = 100 per group). *$p < 0.05$ indicates the significant difference between indicated groups. Scale bar, 100 μm. **(A)** Dex and/morin did not induce toxicity. Myotubes were treated as mentioned above and LDH test was performed as described in section 2.5. Results are mean ± standard deviations, n = 5 per group. NS, not significant. **(B)** Myotubes were treated as described above, followed by the measurement of protein levels for the assessment of fast-type MyHC and slow-type MyHC. The results are expressed as means ± SD ($n = 3$ per group). *$p < 0.05$ indicates the significant difference between indicated groups. NC, negative control with mouse liver sample; PC, positive control with sedentary C2C12 myotubes. MMSTD, molecular mass standard. **(C)** The mRNA expression of the ubiquitin ligases MAFbx1/atrogin-1, MuRF-1, Cbl-b and KLF15. The results are presented as means ± SD (n = 3). *$p < 0.05$ indicates the significant difference between indicated groups.

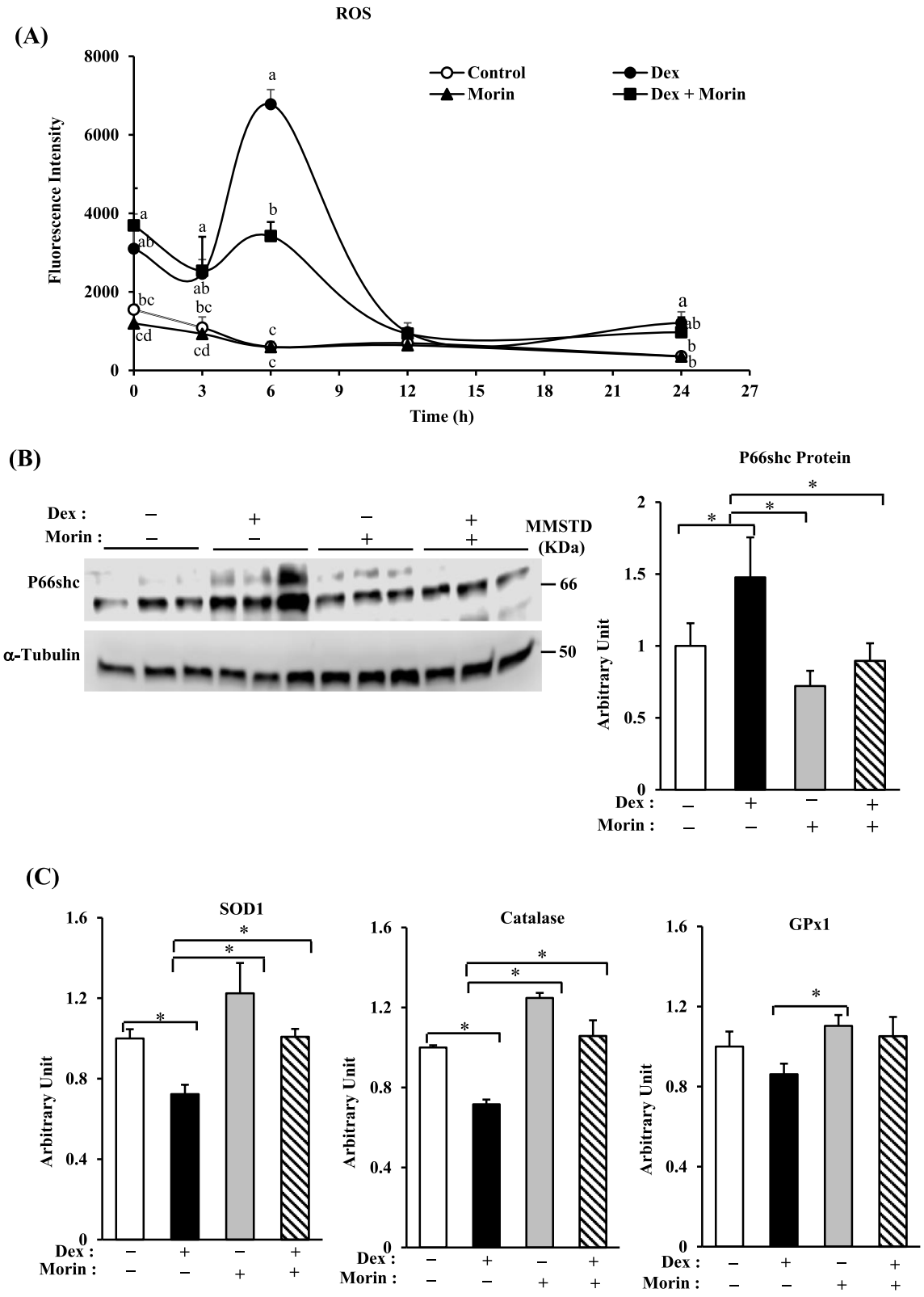

Fig. 2. Morin reduced the Dex-induced oxidative stress in C2C12 myotubes (A) Dex induced ROS production. Myotubes were pretreated with morin for 1 h, followed by Dex treatment for different times (0, 3, 6, 12, and 24 h). ROS production was measured as discussed in subsection 2.8. The results are presented as means ± SD (n = 3). The groups indicated with different letters are significantly different from each other ($p < 0.05$). **(B)** The level of the P66shc protein. The results are presented as means ± SD (n = 3). *$p < 0.05$ indicates the significant difference between indicated groups. MMSTD, molecular mass standard. **(C)** The mRNA expression of SOD1, catalase, and GPx1. The results are expressed as means ± SD (n = 3 per group). *$p < 0.05$ indicates the significant difference between indicated groups.

with the vehicle-treated control. Morin marginally affected the expression of these antioxidant enzymes in C2C12 myotubes with or without Dex treatment. Notably, morin prevented the Dex-mediated suppression of the expression of SOD1 and catalase. These results suggest that morin functions as an antioxidant to suppress Dex-induced atrophy in C2C12 myotubes.

3.4. Effect of morin on muscle-atrophy-associated transcriptional factors or cofactors in C2C12 myotubes

We reported previously that the ROS-induced Cbl-b ubiquitin ligase leads to muscle atrophy by downregulating IGF-1 signaling [11,12]. Because increased dephosphorylation of Foxo3a, which is a downstream molecule of IGF-1 signaling, induces the expression of muscle-atrophy-associated ubiquitin ligases, such as MAFbx1/atrogin-1 and MuRF-1 [21], we examined Foxo3a phosphorylation in C2C12 myotubes treated with Dex in the presence or absence of morin (Fig. 3A). Dex increased total Foxo3a expression in C2C12 myotubes compared with control myotubes, while it significantly decreased the levels of phosphorylated Foxo3a. Interestingly, morin effectively reversed the increase in Foxo3a and the decrease in phosphorylated Foxo3a levels induced by Dex in C2C12 myotubes.

Glucocorticoids induce muscle-fiber type switching from fast-type to slow-type [22]. Morin treatment increased the level of slow-type MyHC in C2C12 myotubes (Fig. 1B). Therefore, we also examined the changes in PGC-1α expression, which is a co-transcriptional factor associated with the induction of slow-type myofibers [23,24]. Although Dex did not alter the levels of PGC-1α protein compared with vehicle-treated control myotubes, treatment with morin alone or in combination with Dex significantly increased PGC-1α levels compared with Dex and/or vehicle-treated myotubes. The morin alone-treated myotubes displayed significantly enhanced expression of PGC-1α (Fig. 3B). This upregulation of PGC-1α by morin is consistent with the increase in slow-type MyHC observed in morin-treated C2C12 myotubes.

3.5. Oxidative stress in Dex-treated myotube atrophy

To confirm the possible association between ROS and Dex-induced muscle atrophy, we examined the effect of GR knockdown on ROS accumulation in C2C12 myotubes using the siRNA technique. GR siRNA transfection reduced GR mRNA expression by almost 60%, whereas the protein levels of GR in the transfected myotubes decreased to 20% of that detected in the control myotubes (Fig. 4A). Furthermore, knockdown of GR also significantly suppressed the mRNA expression of KLF15 (Fig. 4B), a direct transcriptional target of GR. ROS levels was markedly increased in NT siRNA transfected C2C12 myotubes treated with Dex, as indicated by the increased fluorescence, whereas Dex failed to induce ROS in GR-knockdown C2C12 myotubes (Fig. 4C). We also found that Dex did not increase the level of P66shc in GR-knockdown myotubes, but P66shc level was increased in NT siRNA myotubes (Fig. 4D). These results indicate that there is no accumulation of Dex-mediated oxidative stress in GR-knockdown myotubes. Subsequently, the diameter of NT siRNA- and GR siRNA-transfected C2C12 myotubes after Dex and/or morin treatment was measured (Fig. 4E). Dex significantly reduced the diameter of myotubes transfected with NT siRNA. Interestingly, the reduction of myotube diameter was attenuated by morin pretreatment. Myotubes transfected with GR siRNA were unaffected by Dex treatment and morin did not show any effect in the absence of GR. These results

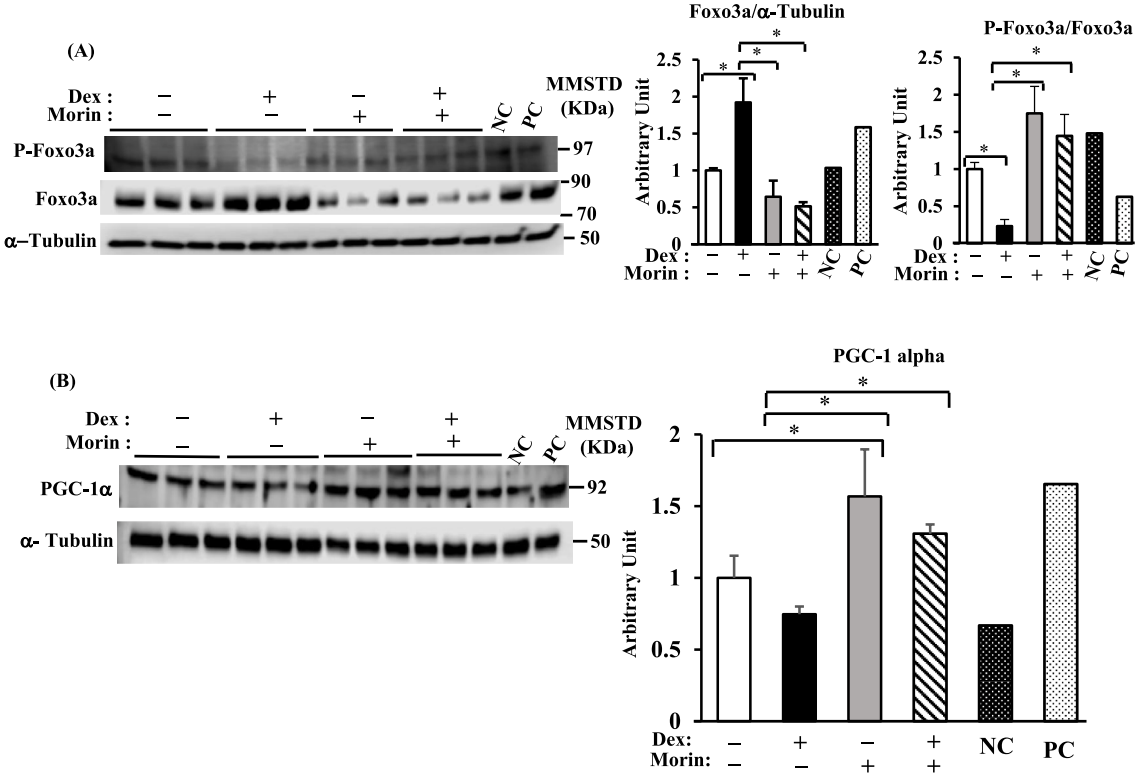

Fig. 3. Morin prevented the Dex-induced upregulation of Foxo3a and increased the PGC-1α. Myotubes were treated as discussed in Fig. 1, followed by the measurement of total and phosphorylated Foxo3a by western blotting. **(A)** Western blot, with the protein levels of Foxo3a and phosphorylated Foxo3a. The results are expressed as means ± SD ($n = 3$ per group). Skeletal muscle samples of sham-operated and sciatic nerve-denervated mice were used as negative controls (NC) and positive controls (PC), respectively, for the analysis of total Foxo3a and phosphorylated Foxo3a (P-Foxo3a) protein levels. *$p < 0.05$ indicates the significant difference between indicated groups. MMSTD, molecular mass standard. **(B)** Morin upregulated PGC-1α, independent form Dex-induced myotubes. Myotubes were treated as discussed in Fig. 1, followed by the measurement of PGC-1α by Western blot. The results are expressed as means ± SD (n = 3 per group). Skeletal muscle samples of sedentary and exercise-induced mice were used as negative controls (NC) and positive controls (PC), respectively, for the analysis of PGC-1α protein levels. *$p < 0.05$, indicates the significant difference between indicated groups. MMSTD, molecular mass standard.

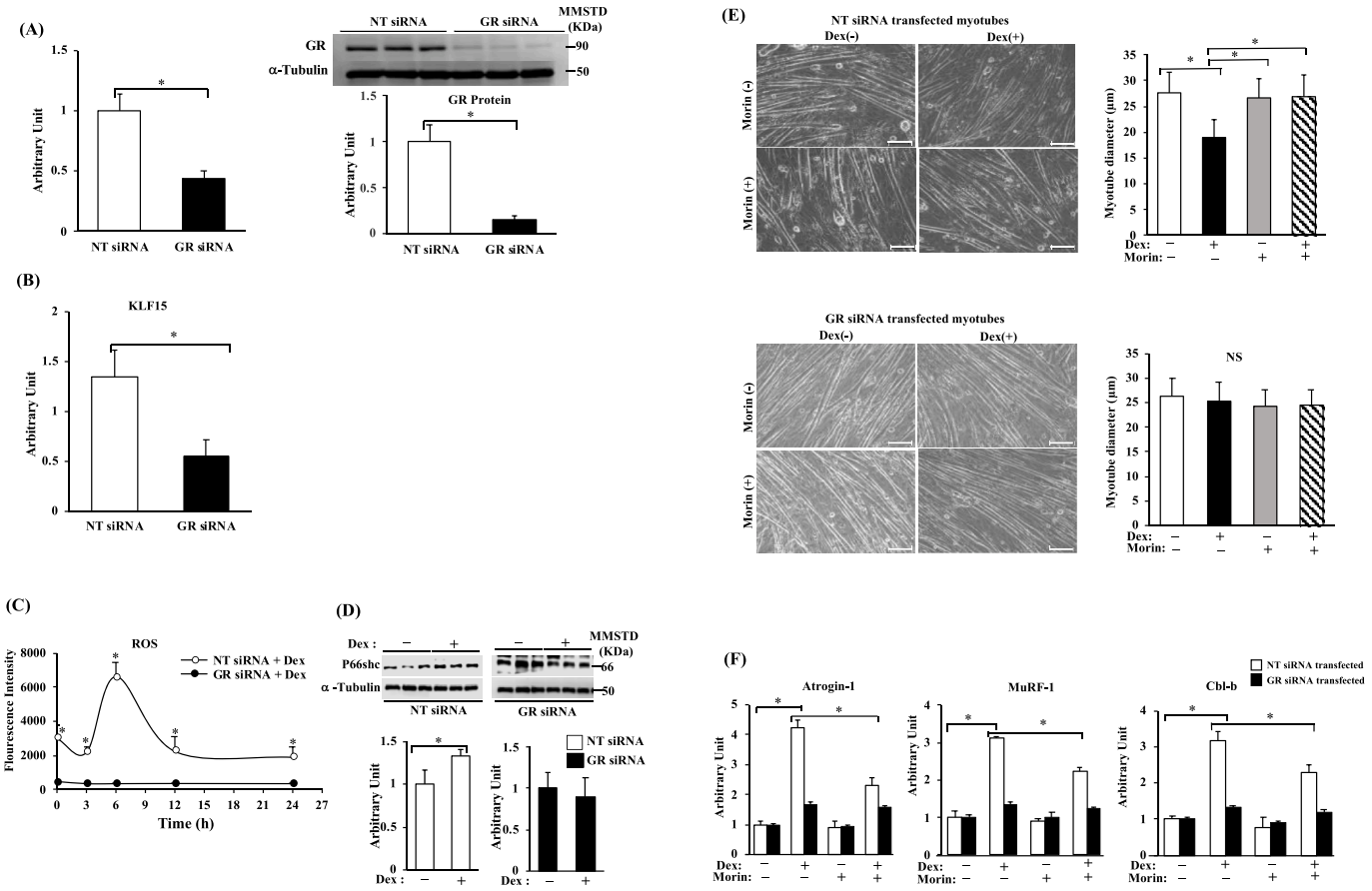

Fig. 4. Dexamethasone produces ROS and muscle atrophy via glucocorticoid receptor (GR). (A) Myotubes were transfected with small interfering glucocorticoid receptor RNA (GR siRNA) and small interfering non-targeting RNA (NT siRNA) as described in the Materials and Methods section, followed by analysis of GR mRNA and protein levels. The results are expressed as means ± SD ($n = 3$). *$p < 0.05$ indicates the significant difference between indicated groups. **(B)** GR knockdown significantly suppressed the mRNA expression of KLF15. The results are expressed as means ± SD ($n = 3$). *$p < 0.05$ indicates the significant difference between indicated groups. **(C)** knockdown of GR significantly reduced ROS production. Transfected myotubes were treated with dexamethasone (10 μM) for different periods and ROS production was measured over 24 h. The results are expressed as means ± SD (n = 3). *$p < 0.05$ compared to GR siRNA-transfected group at respective time points. **(D)** P66shc protein expression in NT and GR knockdown myotubes. Transfected myotubes were treated with Dex or vehicle for 24 h and P66shc levels were measured. The results are expressed as means ± SD (n = 3). *$p < 0.05$ indicates the significant difference between indicated groups. MMSTD, molecular mass standard. **(E)** The presence or absence of GR modifies the atrophic effect in myotubes in response to Dex and/or morin treatment. Transfected myotubes were treated with Dex in the presence or absence of morin as shown in Fig. 1, and the myotube diameter was measured. The results are expressed as means ± SD ($n = 100$). *$p < 0.05$ indicates the significant difference between indicated groups. NS, not significant. Scale bar, 100 μm **(F)** Morin decreased ubiquitin ligase expression induced by Dex in the presence of GR, whereas ubiquitin ligase expression was not affected by Dex or morin in the absence of GR. Transfected myotubes were treated as shown in Fig. 1, followed by the measurement of ubiquitin ligase mRNA expression by qRT-PCR. Results are expressed as means ± SD ($n = 3$ per group). *$p < 0.05$ indicates the significant difference between indicated groups.

suggest that GR is necessary for the increase of ROS levels and development of muscle fiber atrophy in response to Dex treatment. It also indicates that morin is only effective in the presence of GR.

The mRNA expression of ubiquitin ligases was also investigated in NT siRNA- and GR siRNA-transfected myotubes (Fig. 4F). In NT siRNA-transfected myotubes, Dex induced the expression of muscle-atrophy-associated ubiquitin ligases MAFbx1/atrogin-1, MuRF-1, and Cbl-b. The increased expression of these ubiquitin ligases was significantly reduced by morin pre-treatment. In GR siRNA-transfected myotubes, Dex did not significantly increase the expression of ubiquitin ligases; in the absence of GR, morin was found to be ineffective in regulating ubiquitin ligase expression. These findings suggested that the accumulation of oxidative stress was associated with Dex-induced muscle fiber atrophy, which is itself dependent on GR.

4. Discussion

Oxidative stress is linked to muscle atrophy caused by various conditions [25,26]. In this study, we confirmed the relationship between Dex-induced muscle atrophy and oxidative stress. We clearly showed that Dex-induced muscle atrophy was associated with ROS accumulation, which was mediated by the GR, since Dex failed to increase the expression of muscle-atrophy-associated ubiquitin ligases and myotube fiber atrophy in GR-knockdown C2C12 myotubes (Fig. 4). Several previous studies have reported increased ROS accumulation upon Dex treatment. Espinoza et al. showed that glucocorticoid treatment upregulates NADPH oxidase (NOX) mRNA expression, which generates ROS by catalyzing the transfer of electrons to O_2, producing superoxide or H_2O_2, using NADPH as an electron donor [27,28]. This process was independent of the function of GR as transcription factor. These data suggest that the protein-protein interaction between GR and NOX contributes to ROS accumulation upon Dex treatment. Although the detailed function of GR is still controversial, GR has been shown to be necessary for glucocorticoid-mediated oxidative stress and hence muscle atrophy as detailed above.

In this study, we selected morin and examined its effect regarding the attenuation of muscle atrophy induced by glucocorticoid (Dex). Canonically, glucocorticoid (Dex) induces muscle atrophy via direct upregulation of the transcription factor KLF15, which increases the expression of MAFbx1/atrogin-1 and MuRF-1 [2]. In our study, Dex significantly upregulated KLF15. However, morin failed to suppress this Dex-induced KLF15 upregulation, although it significantly prevented Dex-mediated myotube atrophy. Thus, we suggest that morin functions as an antioxidant that targets oxidative stress to prevent Dex-mediated muscle atrophy.

In our study, morin significantly suppressed the Dex-induced ROS accumulation and P66shc expression in C2C12 myotubes. Furthermore, morin pretreatment effectively attenuated the Dex-induced reduction in myotube thickness, as well as the expression of the muscle atrophy-associated ubiquitin ligases MAFbx1/atrogin-1, MuRF-1, and Cbl-b. Morin is a potent ROS scavenger [29]. Data demonstrating changes in ROS levels after Dex and/or morin treatment (Fig. 2A) revealed that the antioxidative activity of morin is directly mediated by ROS scavenging as ROS levels were attenuated coincident with morin treatment. The indirect effect of morin did not significantly contribute to the suppression of oxidative stress, because the increased expression of antioxidative enzymes via Nrf 2, a major transcriptional factor, is a cyto-protective reaction against oxidative stress (i.e., it is necessary for adaptation to oxidative stress conditions).

Morin (2′,3,4′,5,7-pentahydroxyflavone) and quercetin (3,3′,4′,5,7-pentahydroxyflavone) are isomeric antioxidant flavonols. Quercetin has been identified as an effective anti-atrophic agent in dexamethasone-, obesity-, and disuse-induced skeletal muscle atrophy [17,18,30] due to its anti-inflammatory and antioxidant properties, whereas morin has shown significant anti-atrophic effect in cachexia-induced muscle atrophy [31]. The presence of adjacent hydroxyl groups on positions 3′ and 4’ (*ortho*-dihydroxyl) of quercetin provides more stability and antioxidative effect than groups present at the 2′ and 4’ (*meta*-dihydroxyl) positions in morin. Although the antioxidant capacity of morin is weaker than that of quercetin, morin retains its antioxidant character even at higher concentrations, whereas other polyphenols, including quercetin, can produce ROS by undergoing an auto-oxidation reaction [32,33]. It has also been reported that high doses of quercetin have harmful effects in murine bone marrow [34], whereas morin is considered relatively safe even at higher chronic dose administration in rats [35]. Moreover, the absorption of morin in the intestine was found to be 3-fold higher than that of quercetin [36]. Thus, morin seems to be a more beneficial polyphenol compared with quercetin for the prevention of muscle atrophy. Although the precise structure–activity relationships of quercetin and morin with respect to muscle atrophy were not studied, we suggest that the differences of bioactivities between morin and quercetin are due to their structural differences.

Muscle atrophy caused by glucocorticoids is characterized by the transition of muscle fibers from the fast type to the slow type [1,22]. Namely, the fast-type MyHC protein is more dominantly affected by glucocorticoids than is the slow-type MyHC protein. In fact, Dex treatment significantly decreased the fast-type MyHC protein and exerted a lesser effect on slow-type MyHC, whereas morin decreased the Dex-induced fast-type MyHC levels in the present study. Interestingly, morin *per se* significantly increased the level of slow-type MyHC, although it hardly changed the expression of fast-type MyHC. We observed a significant upregulation of PGC-1α by morin compared with Dex and vehicle treated myotubes. As PGC-1α activates the fast-to-slow fiber transformation and mitochondrial biogenesis [23,37], we suggest that the activation of PGC-1α contributes to the increase of slow-type MyHC by morin in the absence or presence of Dex. Unfortunately, the morin-mediated activation of PGC-1α cannot explain its beneficial effect on fast-type MyHC. Further examination is necessary to elucidate this issue.

As summarized in Fig. 5, in this study, we investigated the effectiveness of morin in Dex-induced muscle atrophy using mouse C2C12 skeletal myotubes. Interestingly, we found that morin significantly suppressed the Dex-mediated muscle atrophy and ROS production in C2C12 myotubes, whereas it failed to alter the expression of KLF15 in Dex-treated C2C12 myotubes. Therefore, we suggested that morin

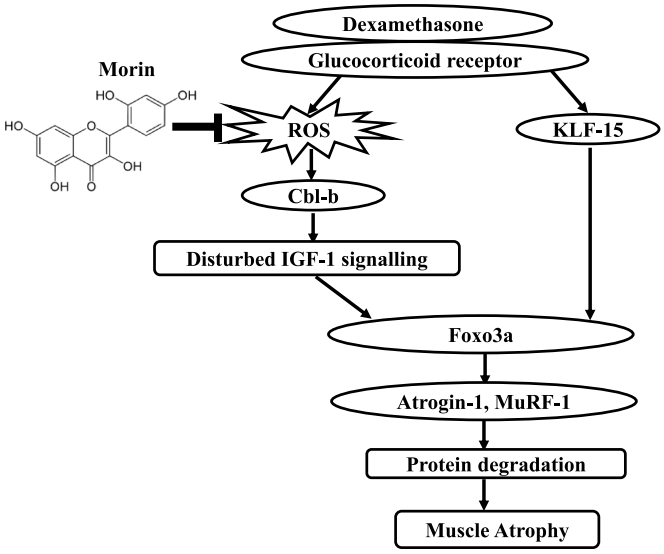

Fig. 5. Diagram of the anti-atrophic effect of morin on dexamethasone-induced muscle atrophy. Dexamethasone induces ROS production, resulting in Cbl-b upregulation, which further dephosphorylates Foxo3a and causes protein degradation and muscle atrophy [12]. By reducing oxidative stress, morin efficiently prevented the muscle atrophy caused by dexamethasone.

exerted the beneficial effects due to effect of suppressing muscle atrophy by decreasing ROS production, at least in part. Muscle atrophy is a severe limiting factor in diseases that require glucocorticoid treatment, such as cancers and autoimmune diseases. In conclusions, this study provides evidence that morin pretreatment diminishes the side effects of glucocorticoid treatment.

Author contributions

T.N:Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition; A.U:Conceptualization, Methodology, Investigation, Data curation, Writing – original draft; T.U: Validation and Formal analysis, Methodology,Y.M:Validation and Formal analysis, Investigation, K.S:Validation and Formal analysis, Investigation, Writing – original draft, A.O:Investigation,T.K:Data curation, R.N: Data curation, Writing – review & editing, Visualization, K.H:Writing – review & editing, I.S:Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

None.

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