

## Original Research



# Ursolic acid improves the indoxyl sulfate-induced impairment of mitochondrial biogenesis in C2C12 cells

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## ABSTRACT

**BACKGROUND/OBJECTIVES:** Patients with chronic kidney disease (CKD) have a high concentration of uremic toxins in their blood and often experience muscle atrophy. Indoxyl sulfate (IS) is a uremic toxin produced by tryptophan metabolism. Although an elevated IS level may induce muscle dysfunction, the effect of IS on physiological concentration has not been elucidated. Additionally, the effects of ursolic acid (UA) on muscle hypertrophy have been reported in healthy models; however, it is unclear whether UA ameliorates muscle dysfunction associated with chronic diseases, such as CKD. Thus, this study aimed to investigate whether UA can improve the IS-induced impairment of mitochondrial biogenesis.

**MATERIALS/METHODS:** C2C12 cells were incubated with or without IS (0.1 mM) and UA (1 or 2 μM) to elucidate the physiological effect of UA on CKD-related mitochondrial dysfunction and its related mechanisms using real-time reverse transcription-polymerase chain reaction, western blotting and enzyme-linked immunosorbent assay.

**RESULTS:** IS suppressed the expression of differentiation marker genes without decreasing cell viability. IS decreased the mitochondrial DNA copy number and ATP levels by downregulating the genes pertaining to mitochondrial biogenesis (*Pparg1a*, *Nrf1*, *Tfam*, *Sirt1*, and *Mef2c*), fusion (*Mfn1* and *Mfn2*), oxidative phosphorylation (*Cyts* and *Atp5b*), and fatty acid oxidation (*Pal4*, *Acadm*, *Cpt1b*, and *Cd36*). Furthermore, IS increased the intracellular mRNA and secretory protein levels of interleukin (IL)-6. Finally, UA ameliorated the IS-induced impairment in C2C12 cells.

**CONCLUSIONS:** Our results indicated that UA improves the IS-induced impairment of mitochondrial biogenesis by affecting differentiation, ATP levels, and IL-6 secretion in C2C12 cells. Therefore, UA could be a novel therapeutic agent for CKD-induced muscle dysfunction.

**Keywords:** Chronic kidney disease; indoxyl sulfate; ursolic acid; mitochondrial biogenesis

## INTRODUCTION

Chronic kidney disease (CKD) is the gradual loss of kidney function accompanied by kidney damage or a decreased glomerular filtration rate over a period of months to years. Patients with CKD, particularly those undergoing dialysis, have various complications, such as cardiovascular and mineral bone disorders. Patients with CKD also show reduced

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**Conflict of Interest**

The authors declare no potential conflicts of interests.

**Author Contributions**

Conceptualization: Norikura T, Kon A; Funding acquisition: Norikura T, Kojima-Yuasa A; Investigation: Sasaki Y, Tadano H, Mizuno A; Methodology: Norikura T, Sasaki Y; Writing - original draft: Sasaki Y; Writing - review & editing: Kon A, Kojima-Yuasa A, Norikura T.

muscle mass and exercise capacity [1]. Therefore, muscle mass is considered an indicator of the clinical condition of patients with CKD [2]. Sarcopenia, which is defined as the loss of skeletal muscle function, because of decreased muscle mass and strength [3], is highly prevalent in patients with CKD [4] and is a serious problem, as it increases mortality and morbidity [5]. Therefore, maintaining muscle mass and strength is important for improving the prognosis of patients with CKD. Restriction of salts and proteins is a fundamental dietary therapy for CKD patients; in contrast, protein supplementation is a fundamental dietary therapy for patients with sarcopenia [6,7]. Because conflicting dietary therapies are recommended for CKD and sarcopenia, nutritional management has not been well established for individuals with both these complications.

Exercise capacity depends on many factors such as cardiopulmonary function, muscle mass, and mitochondrial function [8-10]. Muscle atrophy in patients with CKD associated with mitochondrial dysfunction [11,12]. Mitochondrial DNA (mtDNA) copy number, an indicator of mitochondrial content, is lower in patients with CKD than in healthy subjects. Furthermore, a decreased mtDNA copy number is a potential risk factor for mortality in patients with CKD [12]. Therefore, prevention and amelioration of mitochondrial dysfunction is important for improving muscle atrophy and decreasing the risk of mortality in patients with CKD.

Patients with CKD have a high concentration of uremic toxins in their blood. Indoxyl sulfate (IS) is a uremic toxin produced by metabolism of tryptophan in a diet. IS accumulates in the blood of patients with CKD and impairs the functioning of the liver, thyroid gland, and kidneys [13,14]. IS can cause muscle atrophy in CKD mice through mitochondrial dysfunction [15]. However, the mechanisms underlying IS-induced mitochondrial dysfunction in muscles have not been well elucidated.

Ursolic acid (UA) is a natural triterpene compound found in various fruits and vegetables, such as apples and olives, respectively [16]. UA has a variety of physiological activities, including anti-hyperglycemic [17], anti-inflammation [18], and anti-cancer [19] activities. In addition, UA has shown exercise-mimicking effects, such as promoting muscle hypertrophy and improving insulin sensitivity, in mice [20]. In humans, oral administration of UA remarkably promotes an increase in muscle strength by resistance training [21]. Although the physiological effects of UA on muscles have been reported in healthy humans and rodents, the effects of UA on muscular atrophy in chronic diseases, such as CKD, have not been well reported. Therefore, the aim of this study was to evaluate the physiological effects of UA on mitochondrial biogenesis in CKD.

## MATERIALS AND METHODS

### Cell culture

C2C12 cells, derived from an immortalized mouse myoblast cell line, were obtained from the Riken Cell Bank (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemical, Osaka, Japan) containing 10% fetal bovine serum (Biowest, Nuaille, France) and 1% penicillin-streptomycin (FUJIFILM Wako Pure Chemical) in a humid environment containing 5% CO<sub>2</sub> at 37°C. The cells were seeded at a density of  $7.5 \times 10^5$ /φ35 mm dish. To promote differentiation, the medium was replaced with DMEM containing 2% horse serum (Sigma-Aldrich, St. Louis, MO, USA) after the cells reached 90–100% confluence. Then, the cells were treated with or without IS (IS potassium

salt; Nacalai Tesque, Kyoto, Japan; 0.1 mM) and UA (Tokyo Kasei Kogyo Co., Tokyo, Japan; 1 or 2 μM) for 6 days. The differentiation medium was replaced every second days.

### Cell viability

The effects of IS and UA on cell viability were measured using a neutral red assay [22]. A neutral red stock solution (0.4% neutral red in water) was diluted to 1:80 (v/v) with phosphate buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan). C2C12 cells were treated with or without IS and/or UA for 6 days. At the end of the incubation period, the cells were incubated with the neutral red solution at 37°C for 30 min. Neutral red was extracted from the cells by adding a mixture of 1% (w/v) acetic acid and 50% (v/v) ethanol, followed by culturing at 20–25°C for 30 min. Finally, absorbance was measured in a microplate reader at 540 nm.

### Reverse transcription and real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted from C2C12 cells using ISOSPIN Cell & Tissue RNA (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. After the RNA concentration was measured, equal amounts of total RNA were reverse-transcribed into cDNA using a PrimeScript™ RT reagent kit (TaKaRa Bio, Shiga, Japan) as per the manufacturer's instructions. SYBR green-based real-time PCR was performed in 96-well plates using TB Green™ Premix Ex Taq II reagents (TaKaRa Bio) and StepOnePlus (Thermo Fisher Scientific, Waltham, MA, USA). The reaction procedure was as follows: 95°C for 30 s, followed by 35 cycles at 95°C for 5 s and 60°C for 30 s. The specificity of the real-time PCR product was demonstrated by melting curve analysis and agarose gel electrophoresis. Relative mRNA expression levels were normalized to that of *β-actin*. The data were obtained using the  $\Delta\Delta C_t$  method. The primer sequences are listed in **Table 1**.

**Table 1.** Primer sequences used in real-time PCR analysis

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>Myod1</i>	ATGGTGTCCCTGGTCTTCAC	GACTTCTGCTCTCCCTTCCC
<i>MyoG</i>	CCCAACCCAGGAGATCATTG	CAGTTGGGCATGTTTCGTC
<i>Mfn1</i>	CACTGCAATCTTCGGCCAGT	TTCTGGATTCTGTATGTGCTTCA
<i>Mfn2</i>	TGATGTGGCCCAACTCCAAG	GTAACATCGATCCCAGGGCTGT
<i>Drp1</i>	TGCCTCAGATCGTCGTAGTG	TCTGGTGAACGTGGACTAGC
<i>Fis1</i>	CAAAGAGGAACAGCGGGACT	CAACAGCCCTCGCACATACTT
<i>Ppargc1a</i>	ACCACAACGATGACCCTCC	GTTGTTTGGCTTGAGCATGT
<i>Nrf1</i>	TCTACCCCTCCAAACCCAAC	GCGGCAGCTCTGAATTAACC
<i>Tfam</i>	CCGTATTGCGTGAGACGAAC	GACAAGACTGATAGACGAGGGGA
<i>Sirt1</i>	GGTAGTTCCTCGGTGCCCTA	ACCAAAGAAGACAATCTCTGGCT
<i>Mef2c</i>	ACTTCATCTGTTCCCGCCTCT	TGCAGTAGATATGCGGCTTGG
<i>IL-6</i>	CCACTTCAAAAGTCGGAGGCTTA	CCAGTTTGGTAGCATCCATCATTTC
<i>Pdk4</i>	ACCGGTTTTACATGAACCGCA	GGCTTGGGTTTCCGCTCTTT
<i>Acadm</i>	GACGGAGCAGCCAATGATGT	GGATCTGGGTTAGAACGTGCC
<i>Cpt1b</i>	GGATGATGGCTACGGGGTCT	AGTTCGGCGATGTCCAACAG
<i>Cd36</i>	AACCCAGATGACGTGGCAAA	GGCTCCATTGGGCTGTACAAA
<i>Cyts</i>	ATCTCCACGGTCTGTTCCGGG	TCTCCCCAGGTGATGCCTTT
<i>Atp5b</i>	CTCAGAGGTGTCTGCCTTATTGG	CAGTCAGGTCATCAGCAGGC
<i>ND1</i>	CTAGCAGAAACAAACCGGGC	CCGGCTGCGTATTCTACGTT
<i>b-actin</i>	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCTGCTTGCTG

PCR, polymerase chain reaction; *Myod1*, myogenic differentiation 1; *MyoG*, myogenin; *Mfn1*, mitofusin 1; *Mfn2*, mitofusin 2; *Drp1*, dynamin 1-like; *Fis1*, fission, mitochondrial 1; *Ppargc1a*, peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; *Nrf1*, nuclear respiratory factor 1; *Tfam*, transcription factor A, mitochondrial; *Sirt1*, sirtuin 1; *Mef2c*, myocyte enhancer factor 2C; *IL-6*, interleukin 6; *Pdk4*, pyruvate dehydrogenase kinase, isoenzyme 4; *Acadm*, acyl-Coenzyme A dehydrogenase, medium chain; *Cpt1b*, carnitine palmitoyltransferase 1b; *Cd36*, CD36 molecule; *Cyts*, cytochrome c, somatic; *Atp5b*, ATP synthase, H<sup>+</sup> transporting mitochondrial F1 complex, beta subunit; *ND1*, NAD(P)H dehydrogenase 1; *b-actin*, beta-actin.

### Western blotting

Cells were rinsed with cold PBS and lysed with radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktails (Nacalai Tesque). Equal amounts of proteins were electrophoresed in 10% sodium dodecyl sulphate-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane using a semi-dry blotting apparatus. Membranes were blocked with 1% skimmed milk in Tris-buffered saline-Tween overnight and incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA; anti-phospho-extracellular signal-regulated kinase (ERK) 1/2, anti-ERK1/2, anti-phospho-AKT, anti-AKT, anti-phospho-STAT3, anti-STAT3, and  $\beta$ -actin) for 2 h, followed by incubation with an HRP peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h. After several washing steps, immunodetection was performed using EzWestLumi plus (ATTO Co., Tokyo, Japan).

### mtDNA copy number

Total DNA was extracted from C2C12 cells using a NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The mtDNA copy number was defined as mtDNA (*ND1*): nuclear DNA (*b-actin*), and was determined by real-time PCR, as described in the section "Reverse transcription and real-time quantitative polymerase chain reaction (PCR)."

### ATP levels

C2C12 cells were seeded at a density of  $7.5 \times 10^5$  cells/well in a 96-well plate. After the cells grew to 90%~100% confluence, they were incubated with or without IS (0.1 mM) and UA (1 or 2  $\mu$ M) for 6 days. Following incubation, ATP levels were measured using the CellTiter-Glo<sup>®</sup> 2.0 assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

### Interleukin (IL)-6 secretion measurements

After C2C12 cells were incubated with or without IS (0.1 mM) and UA (1 or 2  $\mu$ M) for 4 days, the culture medium was collected. The concentration of IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) using a mouse IL-6 uncoated ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

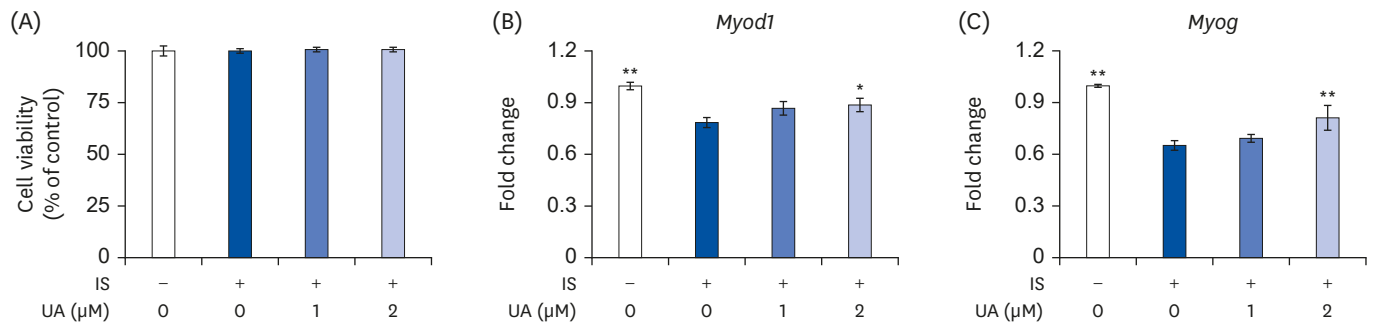
### Statistical analysis

Data are presented as the means  $\pm$  SD of 3 or 6 independent experiments. Comparisons between multiple groups were performed using one-way analysis of variance followed by Dunnett's multiple comparison *post hoc* test.  $P < 0.05$  was considered significant. Statistical analysis was performed using Statcel-4 (OMS Inc., Tokorozawa, Japan).

## RESULTS

### Effects of IS and UA on the cell viability and differentiation of C2C12 cells

The IS concentration increases up to 0.2 mM in the blood of patients with CKD [23]. A recent study reported that IS decreased the viability of C2C12 cells at pharmacological concentrations ( $> 1$  mM) [24]. However, it is unclear whether IS decreases cell viability at physiological concentrations in patients with CKD. As shown in **Fig. 1A**, there was no significant difference in cell viability between the groups. To examine the differentiation state of the C2C12 cells, we measured the mRNA levels of *Myod1* and *Myog*, which are well-known differentiation markers [25]. As shown in **Fig. 1B**, the mRNA expression levels of *Myod1* and *Myog* were significantly

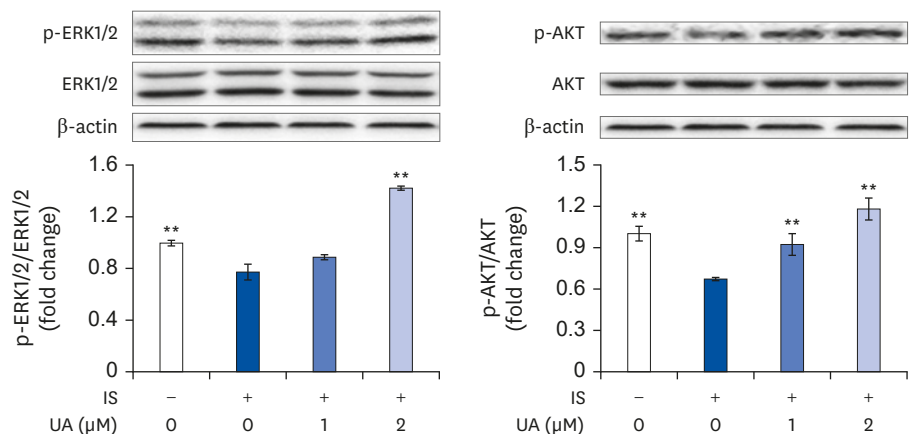


**Fig. 1.** Effects of IS and UA on the cell viability and differentiation of C2C12 cells. C2C12 cells were treated with or without IS (0.1 mM) and UA (1 or 2 μM) for 6 days. (A) Cell viability was assessed using a neutral red assay. (B) The mRNA expression of differentiation marker genes (*Myod1* and *Myog*) was measured by real-time RT-PCR. Data are expressed as mean ± SD (n = 6). IS, indoxyl sulfate; UA, ursolic acid; RT-PCR, reverse transcription-polymerase chain reaction. Each group was compared with the IS-treated group by one-way analysis of variance followed by Dunnett's test, \*P < 0.05 and \*\*P < 0.01.

decreased after treatment with IS; however, this decrease was significantly improved after treatment with UA. Thus, IS suppressed the differentiation of C2C12 cells without decreasing cell viability, and this suppression was ameliorated by treatment with UA.

### UA improved the IS-induced reduction of ERK1/2 and AKT activities in C2C12 cells

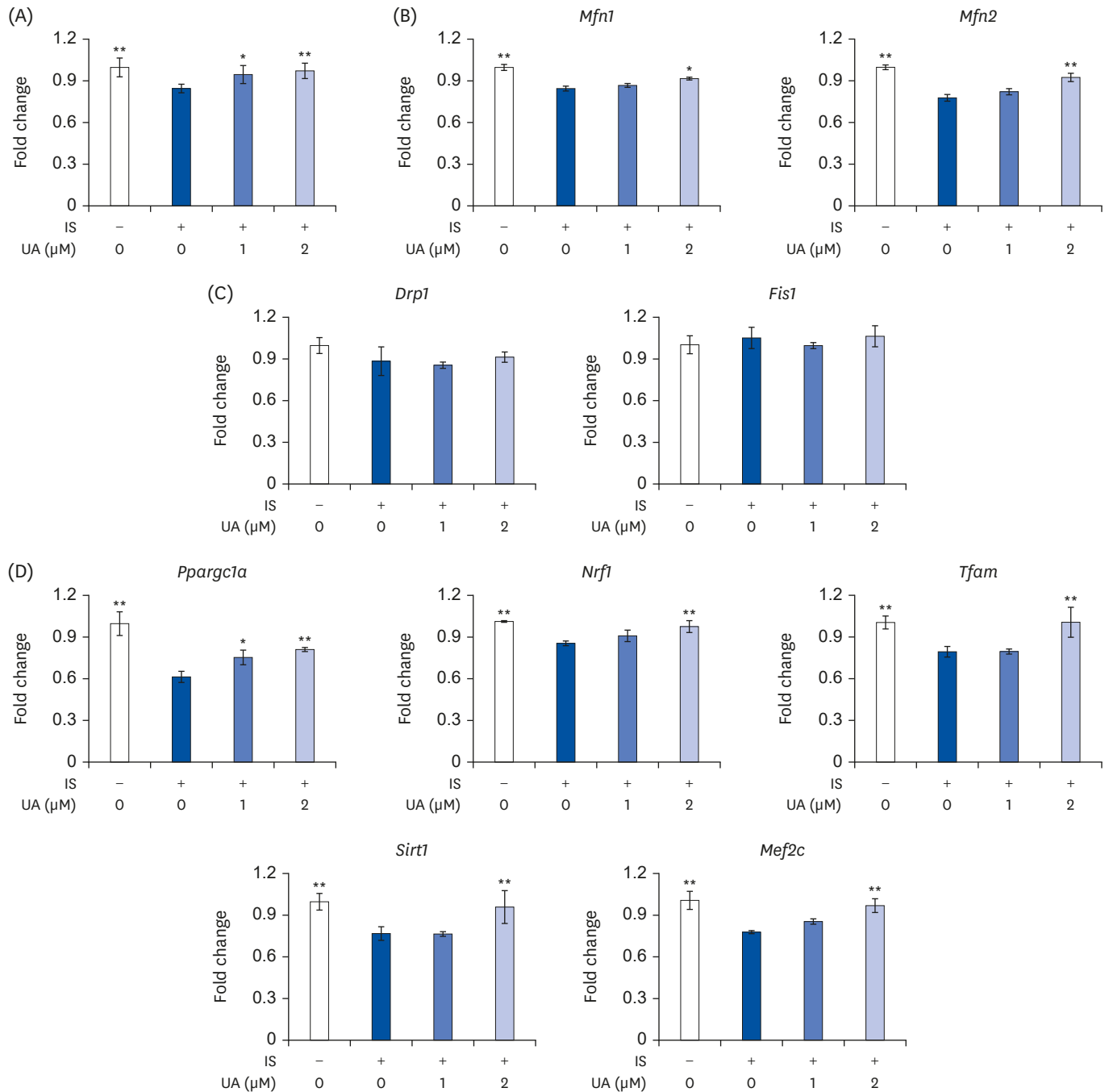
To elucidate the mechanisms of IS and UA in the differentiation of C2C12 cells, we assessed the activities of ERK1/2 and AKT. ERK1/2 and AKT activities are stimulated by more than 100 folds upon phosphorylation of the activation loop residues Thr202/Tyr204 and Ser473, respectively [26,27]. The extent of Thr202/Tyr204 and Ser473 phosphorylation indicates the ERK1/2 and AKT activities. As shown in **Fig. 2**, ERK1/2 and AKT activities were significantly decreased by treatment with IS; however, this decrease was improved after treatment with UA in a dose-dependent manner. Perhaps, IS and UA regulate differentiation through ERK1/2 and AKT activities in C2C12 cells.



**Fig. 2.** Effects of IS and UA on ERK1/2 and AKT phosphorylation. Cells were treated with or without IS (0.1 mM) and UA (1 or 2 μM) for 1 h. The levels of ERK1/2, AKT, and their phosphorylated states were assessed by western blotting. β-actin was used as the internal control. Results are expressed as mean ± SD (n = 3). IS, indoxyl sulfate; UA, ursolic acid; ERK, extracellular signal-regulated kinase; p-, phospho-. Each group was compared with the IS-treated group by one-way analysis of variance followed by Dunnett's test, \*\*P < 0.01.

**UA improved the IS-induced reduction in mitochondrial contents in C2C12 cells**

To assess the effects of IS and UA on the mitochondrial content in C2C12 cells, we measured the mtDNA copy number using real-time PCR. As shown in Fig. 3A, the mtDNA copy number



**Fig. 3.** Effects of IS and UA on mtDNA copy number and mitochondrial biogenesis and dynamics in C2C12 cells. C2C12 cells were treated with or without IS (0.1 mM) and UA (1 or 2 μM) for 6 days. (A) Using real-time PCR, the mtDNA copy number, defined as the ratio of mitochondrial DNA-encoded *ND1* to nDNA-encoded *b-actin*, was determined. (B, C) The mRNA expression levels of mitochondrial fusion (*Mfn1* and *Mfn2*) and fission (*Drp1* and *Fis1*) marker genes were measured by real-time RT-PCR. (D) The mRNA expression levels of *Pparg1a*, *Nrf1*, *Tfam*, *Sirt1*, and *Mef2c* were measured by real-time RT-PCR. Results are expressed as mean ± SD (n = 6). IS, indoxyl sulfate; UA, ursolic acid; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; nDNA, nuclear DNA; RT-PCR, reverse transcription-polymerase chain reaction. Each group was compared with the IS-treated group by one-way analysis of variance followed by Dunnett's test, \*P < 0.05 and \*\*P < 0.01.

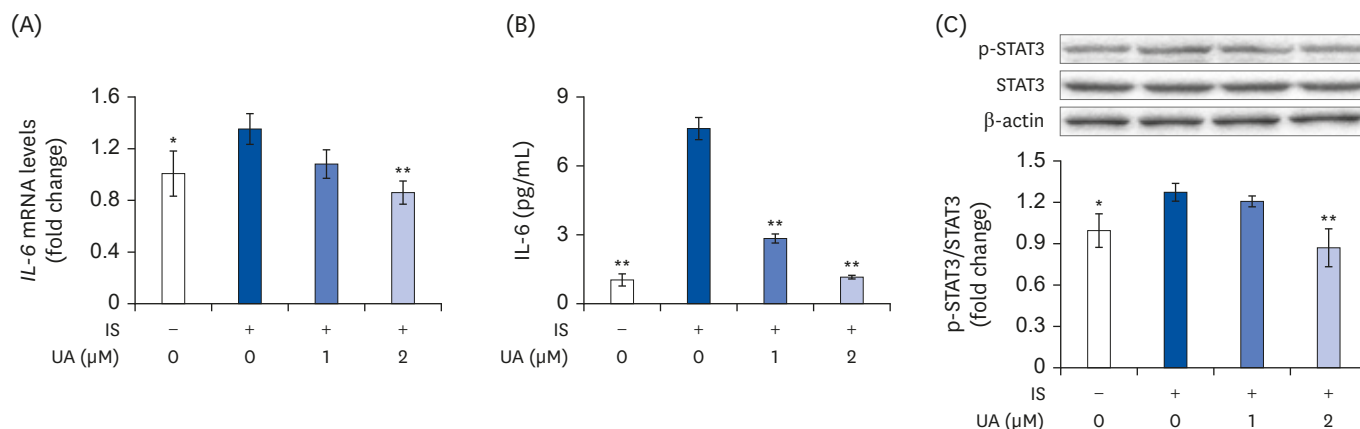
was significantly decreased after the treatment with IS. However, UA significantly improved the IS-induced mtDNA copy number decrease in a dose-dependent manner. Mitochondria undergo fusion and fission, which comprise mitochondrial dynamics, to maintain mitochondrial quality and function after biogenesis. The mtDNA copy number is regulated by mitochondrial dynamics and biogenesis [28]. To assess the effects of IS and UA on mitochondrial dynamics, we investigated the mRNA expression levels of mitochondrial fusion (*Mfn1* and *Mfn2*) and fission (*Drp1* and *Fis1*) marker genes. As shown in **Fig. 3B**, the mRNA expression of *Mfn1* and *Mfn2* was significantly decreased upon the treatment with IS; however, this decrease was improved after treatment with UA. In contrast, there was no significant difference in *Drp1* and *Fis1* expression levels upon treatment with IS (**Fig. 3C**). The mRNA expression levels of mitochondrial biogenesis biomarkers (*Pparg1a*, *Nrf1*, *Tfam*, *Sirt1*, and *Mef2c*) were decreased by treatment with IS; however, the decrease was improved upon treatment with UA (**Fig. 3D**).

### UA improved the IS-induced increase in the mRNA expression and secretion levels of IL-6

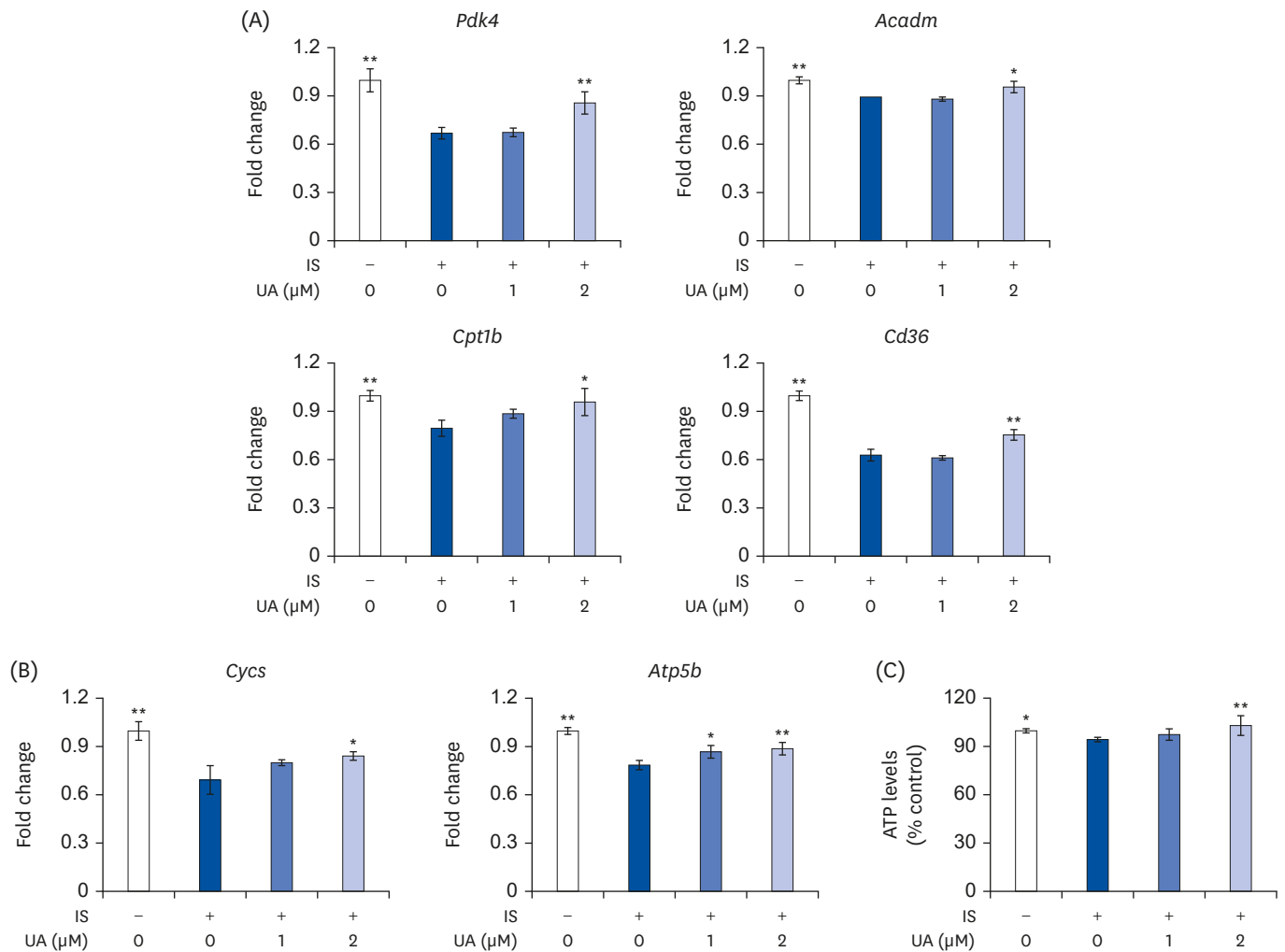
To further investigate the pathways involved in mitochondrial biogenesis, we examined IL-6 expression and secretion levels. As shown in **Fig. 4**, IS increased the intracellular mRNA (**Fig. 4A**) and secretory protein (**Fig. 4B**) levels of IL-6; however, this increase was significantly suppressed by treatment with UA. STAT3 activity is stimulated by the phosphorylation of its activation loop residue of Tyr705 [29]. IL-6 increases STAT3 activity to regulate mitochondrial homeostasis in C2C12 cells. Therefore, we examined the effects of IS and UA on intracellular STAT3 activity. As shown in **Fig. 4C**, STAT3 activity was significantly increased after treatment with IS; however, the increase improved upon treatment with UA in a dose-dependent manner. Perhaps, UA improved mitochondrial biogenesis impairment by suppressing the IS-induced increase in IL-6 levels.

### UA improved the IS-induced reduction in ATP levels in C2C12 cells

The mRNA expression of representative fatty acid oxidation (FAO: *Pdk4*, *Acadm*, *Cpt1b*, and *Cd36*) and oxidative phosphorylation (OXPHOS: *Cyts* and *Atp5b*) genes was downregulated following treatment with IS; however, this was completely reversed with the UA treatment (**Fig. 5A and B**). The ATP levels decreased slightly but significantly with IS treatment; however, this was reversed with the UA treatment (**Fig. 5C**).



**Fig. 4.** Effects of IS and UA on IL-6 expression in C2C12 cells. Cells were treated with or without IS (0.1 mM) and UA (1 or 2 μM) for 4 days. (A) IL-6 mRNA expression was measured using real-time RT-PCR. (B) Secretion levels of the IL-6 protein in the culture media were measured by enzyme-linked immunosorbent assay. (C) The quantified levels of p-STAT3 were normalized to that of STAT3. β-actin was used as the internal control. Data are expressed as mean ± SD (n = 3). IS, indoxyl sulfate; UA, ursolic acid; RT-PCR, reverse transcription-polymerase chain reaction; p-, phospho-. Each group was compared with the IS-treated group by one-way analysis of variance followed by Dunnett's test, \*P < 0.05 and \*\*P < 0.01.



**Fig. 5.** Effects of IS and UA on ATP levels in C2C12 cells. Cells were treated with or without IS (0.1 mM) and UA (1 or 2 μM) for 6 days. (A, B) The mRNA expression levels of *Pdk4*, *Acadm*, *Cpt1b*, *Cd36*, *Cycs*, and *Atp5b* were measured by real-time RT-PCR. (C) The ATP levels in C2C12 cells were assessed using the CellTiter-Glo® 2.0 assay. Data are expressed as mean ± SD (n = 6).

IS, indoxyl sulfate; UA, ursolic acid; RT-PCR, reverse transcription-polymerase chain reaction.

Each group was compared with the IS-treated group by one-way analysis of variance followed by Dunnett's test \* $P < 0.05$  and \*\* $P < 0.01$ .

## DISCUSSION

In this study, we investigated the effects of IS on C2C12 cells to elucidate the mechanisms underlying the muscle dysfunction associated with CKD. This study was conducted with 0.1 mM IS, which is the physiological blood concentration in patients with CKD. Our major findings were as follows: i) IS induced impairment in myoblast differentiation, ii) IS decreased mitochondrial content and ATP levels in C2C12 cells, and iii) IS increased IL-6 secretion from C2C12 cells. In addition, we revealed that UA improved the IS-induced impairments in C2C12 cells.

Nitrogen in dietary protein is mainly metabolized to urea and then excreted in the urine. However, as the excretory function is impaired in patients with CKD, their blood contains higher concentrations of nitrogen-containing urea toxins, such as IS, urea, and creatinine; in fact, the IS concentration can increase up to 0.2 mM in the blood of patients with CKD



[23]. A recent study reported that IS significantly decreased the viability of C2C12 cells a concentration of 1 mM [24]. However, we found that IS (0.1 mM) did not significantly affect the cell viability (**Fig. 1A**). This result suggests that cell death in muscles is not caused by the physiological concentrations of IS in patients with CKD.

Further, muscle strength and exercise capacity decrease in the early stages of CKD [30]. Muscle atrophy is a major complication of CKD and is associated with increased morbidity and mortality. Muscle atrophy is caused by the inactivation of myoblasts and inhibition of differentiation [31]. In this study, IS reduced the mRNA expression of *Myod1* and *Myog* (**Fig. 1B**), which are differentiation markers, in C2C12 cells without decreasing cell viability. These results suggest that IS induced muscular atrophy by suppressing differentiation rather than by causing cell death at physiological concentrations in patients with CKD. Muscle hypertrophy is induced by oral administration of UA [20]. Intraperitoneal administration of UA has been known to promote muscle hypertrophy in combination with low-intensity exercise in rats [32]. However, to our knowledge, the effect of UA on chronic disease-related muscular atrophy has not yet been reported. In this study, UA ameliorated the IS-induced decrease in the expression of differentiation markers (**Fig. 1B**). These results suggest that UA may improve IS-induced muscular atrophy and ameliorate the poor prognosis of patients with CKD.

The activities of ERK1/2 and AKT play pivotal roles in the differentiation of C2C12 cells. 4-hydroxy-3-methoxy cinnamic acid promotes the differentiation of C2C12 cells through an increased expression of *Myod1* and *Myog*. These effects are suppressed upon treatment with ERK1/2 and AKT inhibitors [30]. In this study, the activities of ERK1/2 and AKT were significantly decreased by treatment with IS; however, this decrease was improved upon treatment with UA (**Fig. 2**). These results suggest that ERK1/2 and AKT activities are involved in the regulation of IS and UA in the differentiation of C2C12 cells.

Mitochondria play an important role in myogenesis. As myoblasts differentiate into myotubes, the content and morphology of the mitochondria are altered. For example, mitochondrial content, mass, and volume are remarkably increased after the onset of myoblast differentiation [33]. However, in a CKD mouse model induced by mutations in the *Anks6* gene, the expression levels of *Myod* and *Myog* increased despite muscle atrophy and downregulation of mitochondrial biogenesis [34]. Therefore, to evaluate CKD-related muscle atrophy, it is important to evaluate, not only myoblast differentiation, but also mitochondrial biogenesis. In this study, mtDNA copy number, which is an indicator of mitochondrial content, was decreased on treatment with IS; however, this decrease was improved after treatment with UA (**Fig. 3A**). Previous studies have reported that the mtDNA copy number in the muscles is reduced in patients with CKD stage 5 undergoing hemodialysis [11]. These results suggest that UA possibly suppresses the decrease in the mitochondrial content and improves muscle function in CKD patients.

Mitochondrial dynamics are important for maintaining the overall morphology of mitochondria. Disorders of mitochondrial dynamics are associated with various diseases, including cancer, cardiovascular diseases, and diabetes [35,36]. However, the involvement of mitochondrial dynamics in CKD progression has not been well elucidated. As shown in **Fig. 3B**, we observed that the expression of mitochondrial fusion markers (*Mfn1* and *Mfn2*) decreased upon treatment with IS; however, this decrease was improved after treatment with UA. In contrast, the expression of the fission markers (*Drp1* and *Fis1*) was not significantly

altered after treatment with IS and UA (**Fig. 3C**). Mitochondrial fusion is essential for maintaining the homeostasis of mtDNA, because the deficiency of mitochondrial fusion factors induces accumulation of mtDNA mutations and a reduction in the mtDNA copy number [37]. Although mitochondrial fission is not important for maintaining mtDNA levels, it is involved in the clearance of dysfunctional mitochondria [38,39]. These results suggest that UA reversed the IS-induced reduction in the mtDNA content by maintaining mitochondrial fusion functions. Mitochondrial biogenesis promotes an increase in mitochondrial content to meet intracellular energy demands [40]. Downregulation of mitochondrial biogenesis leads to muscle atrophy and subsequent detrimental consequences for exercise capacity in patients with CKD [41,42]. Mitochondrial biogenesis is activated by the expression of mitochondrial and nuclear genes that are regulated by transcription factors, such as *Pparg1a*, *Nrf1*, *Tfam*, *Sirt1*, and *Mef2c*. In particular, *Pparg1a* is a well-known pivotal regulator of mitochondrial biogenesis. In this study, the mRNA expression levels of *Pparg1a*, *Nrf1*, and *Tfam* were decreased upon treatment with IS; however, this decrease was improved upon treatment with UA (**Fig. 3D**). *Pparg1a* promotes the transcriptional activity of *Tfam* by binding to and co-activating *Nrf1* on the *Tfam* promoter [43]. *Tfam* activates mtDNA replication and transcription, and the interaction between *Tfam* and mtDNA is involved in the regulation of mitochondrial biogenesis [44]. These results revealed that UA improved the IS-induced reduction in mitochondrial biogenesis and mtDNA content by regulating the expression of *Pparg1a*, *Nrf1*, and *Tfam*. In addition, the expression of *Pparg1a* is mediated by *Sirt1* and *Mef2c* [45]. In this study, the mRNA expression levels of *Sirt1* and *Mef2c* were decreased by treatment with IS; however, the decrease was improved upon treatment with UA (**Fig. 3D**). These results also indicate that UA ameliorates the IS-induced reduction of mitochondrial content in C2C12 cells by regulating the expression levels of the abovementioned genes.

A recent study reported that muscle mitochondrial contents were decreased in CKD mice, and this decrease was related to an increased concentration of the inflammatory cytokine IL-6 in blood [46]. White *et al.* [47] reported that IL-6 overexpression induced muscle atrophy in a mouse model of colon cancer, and this induction was associated with the upregulation of *Fis1* expression and downregulation of *Pparg1a*, *Mfn1*, and *Mfn2* expression. In addition, administration of the IL-6 receptor antibody ameliorates the IL-6-induced decrease in mitochondrial content and expression of *Fis1*, *Pparg1a*, *Mfn1*, and *Mfn2* [47]. In this study, *IL-6* mRNA expression and protein secretion were increased upon treatment with IS; however, this increase was improved after treatment with UA (**Fig. 4A and B**). In addition, STAT3 activity was significantly increased after treatment with IS; however, this increase was improved by treatment with UA (**Fig. 4C**). While IL-6 activates many signaling pathways, the activation of STAT3, which is immediately downstream of IL-6, has been associated with the loss of mitochondrial functions in muscles [48]. These results suggest that UA improved the decrease in mitochondrial content by suppressing the IS-induced increase in IL-6 secretion.

Normal differentiated cells maintain viability and function through ATP production, which relies on mitochondrial OXPHOS. Fatty acid is a central fueling substrate for ATP production in mitochondria. In this study, IS decreased the mRNA expression of representative FAO genes (*Pdk4*, *Acadm*, *Cpt1b*, and *Cd36*); however, this decrease was improved by treatment with UA (**Fig. 5A**). These results indicate that UA contributes to the maintenance of energy production capacity by suppressing the IS-induced decrease in the expression of FAO-related genes. In addition, IS decreased the mRNA expression of representative OXPHOS genes (*Cytc* and *Atp5b*) and intracellular ATP levels; however, this decrease was improved upon treatment

with UA (**Fig. 5B and C**). Intracellular ATP levels increase with the progression of myoblast differentiation to meet increased energy demands [49]. These results suggested that UA maintains normal differentiation by suppressing the decrease in IS-induced ATP levels in C2C12 cells.

In summary, the current study revealed that UA improved the IS-induced impairment in mitochondrial biogenesis, and its mechanisms were associated with muscle differentiation, ATP levels, and IL-6 secretion. Therefore, this study indicated that UA could be a potential therapeutic candidate for treating CKD-induced muscle dysfunction.

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