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Original Article

Sirtuin 3 modulation by high phosphates: a potential mechanism in muscle aging and sarcopenia

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Abstract: Objectives: To investigate the role of elevated phosphate levels in muscle aging, and to elucidate the underlying molecular mechanisms by which high phosphate conditions regulate muscle aging and explore the potential therapeutic role of SIRT3 activation. Methods: Young (5-month-old) and aged (24-month-old) C57BL/6 mice were compared in terms of body weight, muscle strength, and serum sodium levels. Additionally, C2C12 myoblasts were exposed to 20 mM β-glycerophosphate (BGP) to simulate high phosphate conditions. Cellular senescence was assessed using senescence-associated β-galactosidase (SA-β-GAL) staining and Western blot analysis (P53, P62, and P21). The role of SIRT3 in muscle cell senescence was further investigated by treating C2C12 cells with the SIRT3 activator 2-APQC. Results: Aged mice exhibited significantly higher body weight, reduced grip strength, and elevated serum sodium levels compared to young mice, indicating muscle aging. BGP treatment in C2C12 cells induced cellular senescence, as evidenced by elevated SA-β-GAL activity and upregulation of senescence markers P53, P62, and P21. Furthermore, high phosphate levels impaired cell migration and differentiation. Activation of SIRT3 by 2-APQC alleviated these effects, restoring autophagic activity and reversing muscle cell dysfunction. Conclusions: Elevated serum sodium and phosphate levels are associated with muscle aging in mice. High phosphate induces cellular senescence and impairs muscle function, while SIRT3 activation mitigates these effects, highlighting its potential as a therapeutic target for sarcopenia. Dietary phosphate restriction and activation of SIRT3 may represent effective strategies for combating age-related muscle degeneration.

Keywords: Muscle aging, sarcopenia, high phosphate, SIRT3, senescence-associated β-galactosidase, muscle function

Introduction

As the global population ages, senescence-related issues in the elderly have become increasingly prominent [1, 2]. A major concern associated with aging is the loss of muscle strength, leading to reduced muscle mass, impaired motor function, and a decline in overall physical capability. This muscle deterioration severely impacts daily living activities and quality of life [3]. Sarcopenia can contribute to both physical and cognitive decline during aging, further exacerbating underlying diseases and ultimately reducing life expectancy [4]. Muscle tissue senescence is driven by a complex interplay of cellular and molecular mechanisms, including intracellular signaling, gene expression, and protein synthesis [5]. Elucidating these mechanisms is critical for developing effective interventions to improve the quality of life and overall health in the elderly. Although significant progress has been made, many aspects of the molecular mechanisms associated with muscle aging remain unclear.

Senescence is an inevitable process that gradually leads to functional decline in nearly all organisms [6]. Cellular senescence, a hallmark of aging, plays a central role in driving age-related tissue dysfunction [7]. It can be triggered by

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telomere attrition following repeated cell division, or prematurely by various stressors such as oncogene activation, DNA damage, or oxidative stress [8]. Environmental factors also contribute to the induction of senescence. Notably, sodium accumulation in skeletal muscle is significantly higher in the elderly compared to the young [9]. In a Drosophila model, a high salt-phosphate diet promoted muscle senescence via the NAD+/dSir2/dFOXO pathway, leading to increased mortality [10]. Furthermore, extracellular matrix (ECM) content, a known aging phenotype of the skin, was shown to decrease following high salt-phosphates diet (HSD) in mice, which also induced a pro-inflammatory state in the skin, accelerating skin aging [11]. Studies have demonstrated that grip strength and chair elevation tests were significantly lower in aged mice on a high salt-phosphate diet compared to those on a low salt-phosphate diet [12]. These studies collectively suggest a strong association between high salt-phosphates and muscle aging.

Sirtuins, a family of highly conserved NAD+ dependent enzymes, have been extensively studied for their roles in cellular processes. Among them, Sirtuin 3 (SIRT3), a key mitochondrial deacetylase, regulates metabolism-related proteins involved in mitochondrial energy production, stress response, fuel partitioning, and signaling [13]. SIRT3 functions as a metabolic switch, adapting cellular responses to nutrient status and stress conditions [14]. In the liver, high salt-phosphate intake epigenetically suppresses SIRT3, contributing to chronic inflammation and cardiovascular damage [15]. In the heart, SIRT3-iduced mitochondrial dysfunction is a key factor in salt-phosphate induced cardiac hypertrophy [16]. Moreover, an imbalance in mitochondrial homeostasis, driven by SIRT3, has been implicated in cognitive decline, as SIRT3 inhibition under high salt-phosphate conditions exacerbates cognitive impairment by interfering with SIRT3/PINK1-mediated mitochondrial autophagy [17]. In addition, SIRT3 inhibition also downregulates key autophagy markers such as LC3B, Beclin1, and P62 [18]. Despite these findings, the role of SIRT3 in muscle aging remains unclear.

The present study aimed to investigate the molecular mechanisms by which salt-phosphates regulate SIRT3 expression and contributes to muscle cell senescence. We further explored the potential of targeting this pathway as a therapeutic intervention for sarcopenia. Our results demonstrated that high salt-phosphate exposure upregulated senescence markers in myoblasts and impaired muscle cell differentiation by modulating SIRT3 expression.

Materials and methods

Animal study design

Five-month-old and 24-month-old male C57BL6 mice were obtained from Charles River (China). The mice were divided into two groups: the young group (5-month-old, n = 5) and the aged group (24-month-old, n = 5). All mice were housed under controlled conditions (12:12 h light-dark cycle, 24°C) with ad libitum access to standard chow and water.

Following behavioral experiments, orbital blood was collected under anesthesia (ketamine, 100 mg/kg) to minimize pain. The mice were then euthanized by carbon dioxide inhalation, with death confirmed by cervical dislocation. Skeletal muscle tissues, including the extensor, gastrocnemius, piriformis, diaphragm, and tibialis anterior, were harvested for further analysis. Each muscle was carefully dissected, cleaned of connective tissue and blood, and weighted. The relative muscle weight was calculated as follows: relative muscle weight (%) = (muscle weight (mg)/overall body weight (g)) × 100%.

All animal experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the Ethics Committee of Haikou People's Hospital affiliated to Xiangya School of Medicine, Central South University (Approval No. SC20220101).

Grip strength test

Grip strength was measured using the Bioseb grip test (BIO-GS4; Bioseb, USA). Mice were tested for grip strength as previously described [19]. Each mouse was tested three times with a rest interval of 20-40 second between trials. The average grip strength of the three tests was calculated for each mouse.

Cell culture

C2C12 mouse myoblasts were obtained from Procell (China, CL-0044) and cultured in Dulbecco's Modified Eagle medium (DMEM; 11965118, Thermo Fisher, USA) containing 4.5 g/L glucose, 10% fetal bovine serum (A5670-701, Thermo Fisher, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (15140122, Thermo Fisher, USA). The cells were maintained in a cell culture chamber with 95% air and 5% CO_2 at 37°C.

For the hyperglycolate group, cells were treated with 20 mM β-glycerophosphate (BGP; Cayman Chemical, USA), an extracellular phosphate donor [20]. To evaluate the effects of SIRT3 activation, cells were treated with 10 mM of the SIRT3 activator 2-APQC (HY-158426, MCE, USA) under designated experimental conditions.

Detection of senescence-associated β-galactosidase (β-GAL) activity

Senescence-associated β-galactosidase (SA-β-GAL) staining was performed using a commercial kit (I2904, Thermo Fisher, USA) according to the manufacturer's instructions. C2C12 cells were seeded in 6-well plates and subsequently stained for SA-β-GAL activity. Nuclei were counterstained with DAPI (Roche, 10236276001, Switzerland). After staining, cells were cultured in a dry incubator at 37°C.

Stained cells were examined using a LEICA TCS-SP5 confocal microscope (Leica Microsystems, Germany). SA-β-GAL activity was visualized via green fluorescence upon excitation at 488 nm using an argon laser, while DAPI-stained nuclei were observed at 405 nm.

Cell scratch assay

C2C12 cells were cultured in a medium containing 20 mM BGP until reaching full confluence. A 0.6 mm wide scratch was created in the monolayer using a sterile needle. Wound closure was observed at 0, 12, and 24 hours using a Moticam microscope (Motics, USA), and images were captured at each time point. The wound area was then quantified using ImageJ software. The migration rate was calculated using the following formula: Migration Rate (%) = $[(A_{0\ h} - A_{24\ h})/A_{0\ h}] \times 100\%$, where $A_{0\ h}$ represents the initial wound area and $A_{24\ h}$ represents the wound area at 24 hours.

C2C12 differentiation assay

The expression of myosin heavy chain (MHC) and desmin was assessed by immunofluorescence. Following BGP treatment, cells were rinsed twice with PBS and fixed with 4% paraformaldehyde (P0099, Beyotime, China) for 15 minutes at room temperature. Cells were then permeabilized with 0.5% Triton X-100 (P0096, Beyotime, China) for 10 minutes and blocked with 5% bovine serum albumin (V900933, Sigma-Aldrich, USA) for 1 hour at room temperature.

Subsequently, cells were incubated overnight at 4°C with rabbit anti-desmin antibody (1:500, ab32362, Abcam, USA) in a humidified chamber, followed by incubation with mouse anti-MHC antibody (1:500, sc-376157, Santa Cruz Biotechnology, USA) for 2 hours at room temperature. After rinsing with PBS, cells were incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:200; 4412S, Cell Signaling Technology, USA) and Alexa Fluor 647-conjugated goat anti-mouse IgG (1:200; 4410S, Cell Signaling Technology, USA) for 1 hour. Fluorescence images were captured using a confocal microscope (LEICA TCS-SP5, Leica Microsystems, Germany).

Western blot (WB) analysis

Cells and mouse muscle tissues from each group were collected, and proteins were extracted from the cells or tissues. The protein samples were then electrophoresed on 8-12% SDS-PAGE gels under reducing conditions and transferred to a PVDF membrane. The membranes were blocked with 5% skim milk for 1 hour at room temperature, followed by overnight incubation at 4°C with the corresponding primary antibodies. After washing, membranes were incubated with the appropriate secondary antibody for 1 hour at room temperature.

The primary antibodies used were as follows: anti-P53 (1:1000, ab32049, USA), anti-P62 (1:1000, ab207305, USA), anti-SIRT3 (1:1000, ab217319, USA), anti-P21 (1:1000, ab109520, USA), anti-AC-P53 (1:1000, ab75754, USA), anti-LC3B (1:1000, ab19289, USA), and anti-GAPDH (1:1000, ab8245, USA) (all from Abcam,

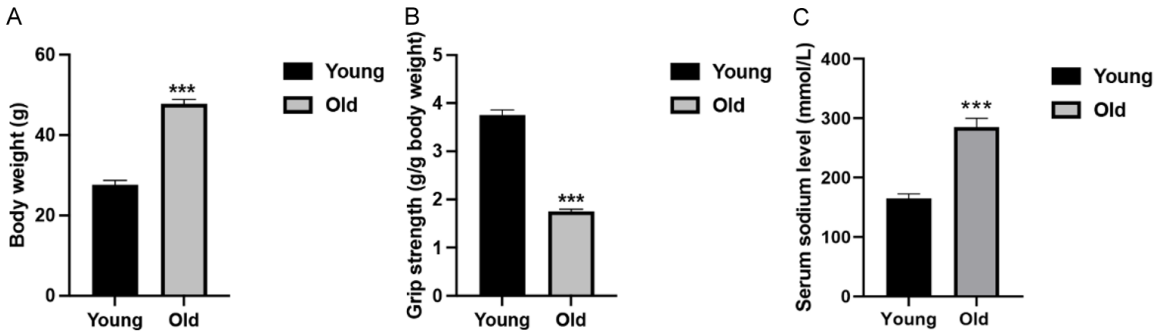

Figure 1. Relationship between sodium levels and muscle aging in mice. A. Body weight. B. Grip strength. C. Serum sodium concentration. ***P < 0.001, n = 5.

USA). The blots were then scanned, and protein bands were detected using a highly sensitive ECL chemiluminescence detection kit (Proteintech, PK10003, China).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 7.0 software (USA). Comparisons between two groups were performed using the unpaired Student's t-test. For multiple group comparisons, one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was applied. A *P*-value of < 0.05 was considered statistically significant. All experiments were independently repeated three times to ensure reproducibility.

Results

Elevated serum sodium levels were associated with muscle aging in mice

To investigate the role of muscle in the aging process, we compared body weight, muscle function, and related biochemical indices between young (5-month-old) and aged (24-month-old) C57BL/6 mice. Aged mice exhibited significantly higher body weights compared to young mice, consistent with typical age-related changes in body composition. Despite the weight gain, the aged mice exhibited significantly reduced grip strength compared to the younger group (**Figure 1A**, **1B**), indicating an age-associated decline in muscle function.

Furthermore, serum sodium levels were significantly elevated in the aged group (**Figure 1C**), suggesting a potential association between increased sodium levels and muscle aging.

Aging significantly reduced muscle mass

Loss of muscle mass is a hallmark of aging. To quantify this, we measured the relative weights of various skeletal muscles, including the extensor digitorum longus, gastrocnemius, soleus, diaphragm, and tibialis anterior. The relative weights of all these muscles were significantly lower in the aged group compared to the young group (**Figure 2A-E**), confirming substantial age-related muscle atrophy.

Aging-related markers were upregulated while SIRT3 expression was suppressed in aged muscle tissues

To further elucidate the molecular alterations associated with aging in muscle tissues, we examined the expression levels of aging-related protein markers, P62 and P53. Western blot analysis revealed significantly elevated levels of both P62 and P53 in the muscle tissues of aged mice compared to young mice (**Figure 3A-C**). P62, an autophagy adaptor protein, accumulates in senescent cells due to impaired autophagic degradation. P53, a key regulator of cell cycle arrest and stress response, is also commonly upregulated in senescent tissues. These findings indicate enhanced cellular senescence and autophagy dysfunction in aged muscle.

In addition, we observed a significant reduction in SIRT3 expression, a critical regulator of mitochondrial homeostasis, in the muscle tissues of aged mice (**Figure 3D**). SIRT3 plays a crucial role in maintaining mitochondrial function and cellular energy balance, and its downregulation has been linked to various age-related pathologies, including muscle degeneration.

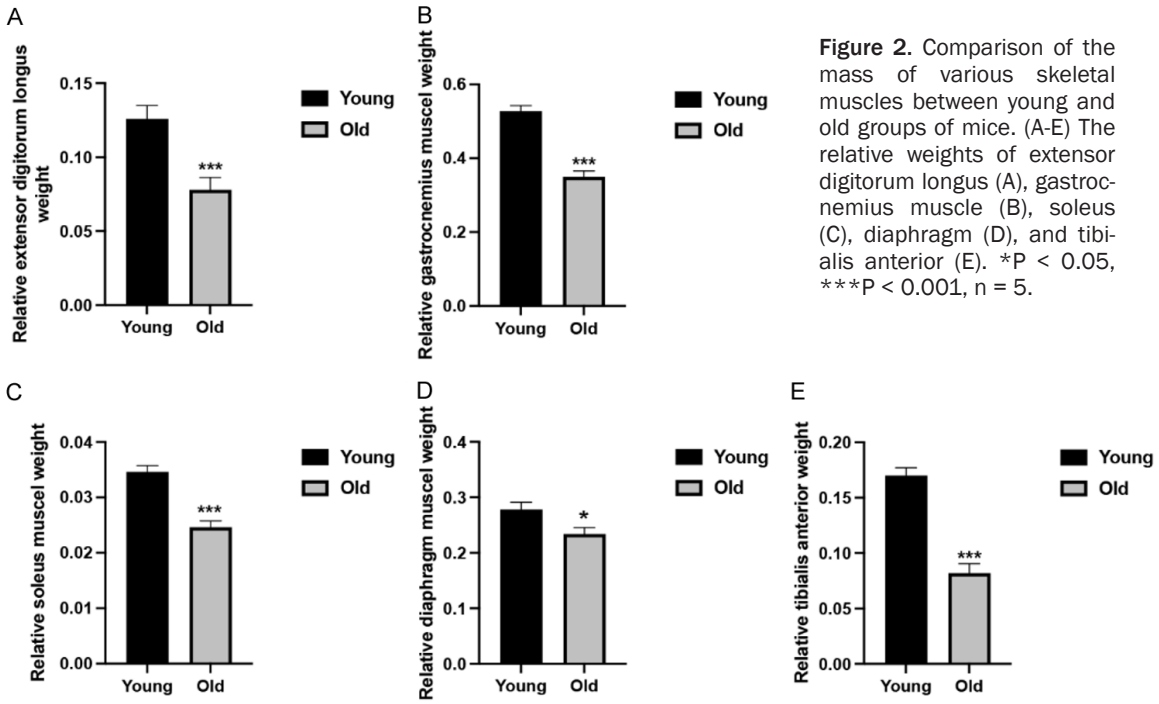

Figure 2. Comparison of the mass of various skeletal muscles between young and old groups of mice. (A-E) The relative weights of extensor digitorum longus (A), gastrocnemius muscle (B), soleus (C), diaphragm (D), and tibialis anterior (E). *P < 0.05, ***P < 0.001, n = 5.

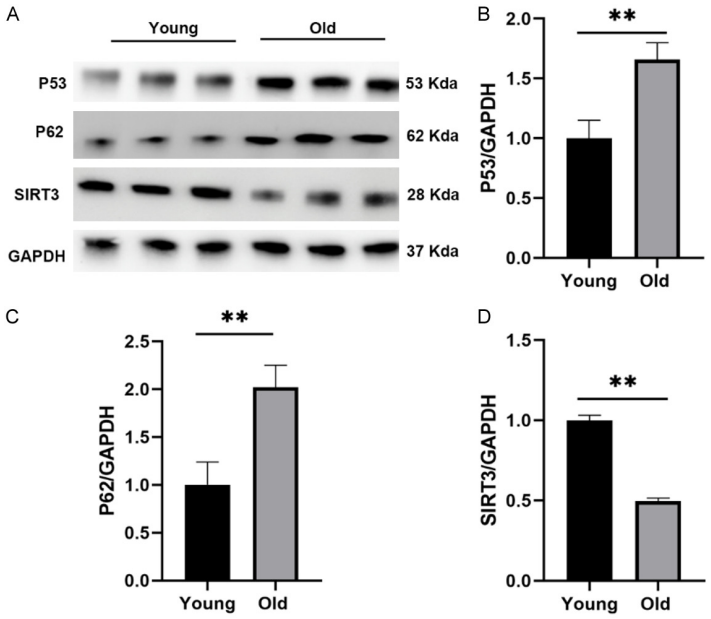

Figure 3. Comparison of senescence markers in muscle tissues between young and aged mice. (A) WB band of senescence markers; (B-D) Relative protein expression of P62 (B), P53 (C) and SIRT3 (D). **P < 0.01, n = 3.

The reduced expression of SIRT3 in aged muscle tissues prompted us to hypothesize that SIRT3 may be closely associated with the degradation of muscle function and disruption of cellular homeostasis during aging.

High phosphate exposure induced senescence in C2C12 cells

To determine whether high phosphate levels directly induce muscle cell senescence, C2C12 myoblasts were treated with 20 mM BGP. Senescence-associated β-galactosidase (SA-β-GAL) staining revealed a significant increase in SA-β-GAL-positive cells in the BGP-treated group compared to controls (**Figure 4A**). This finding suggests that high phosphate levels can trigger the senescence pathway in muscle cells.

Western blot analysis further demonstrated that the expression of key senescence-associated proteins, including acetylated P53 (AC-P53), P53, and P21, was markedly upregulated within 24 to 48 hours of BGP exposure (**Figure 4B**). These proteins are key regulators of the cell cycle arrest and stress responses, and their elevated expression is a hallmark of cellular senescence. Interestingly, their expression levels slightly decreased after 72 to 96 hours of treatment, possibly reflecting an adap-

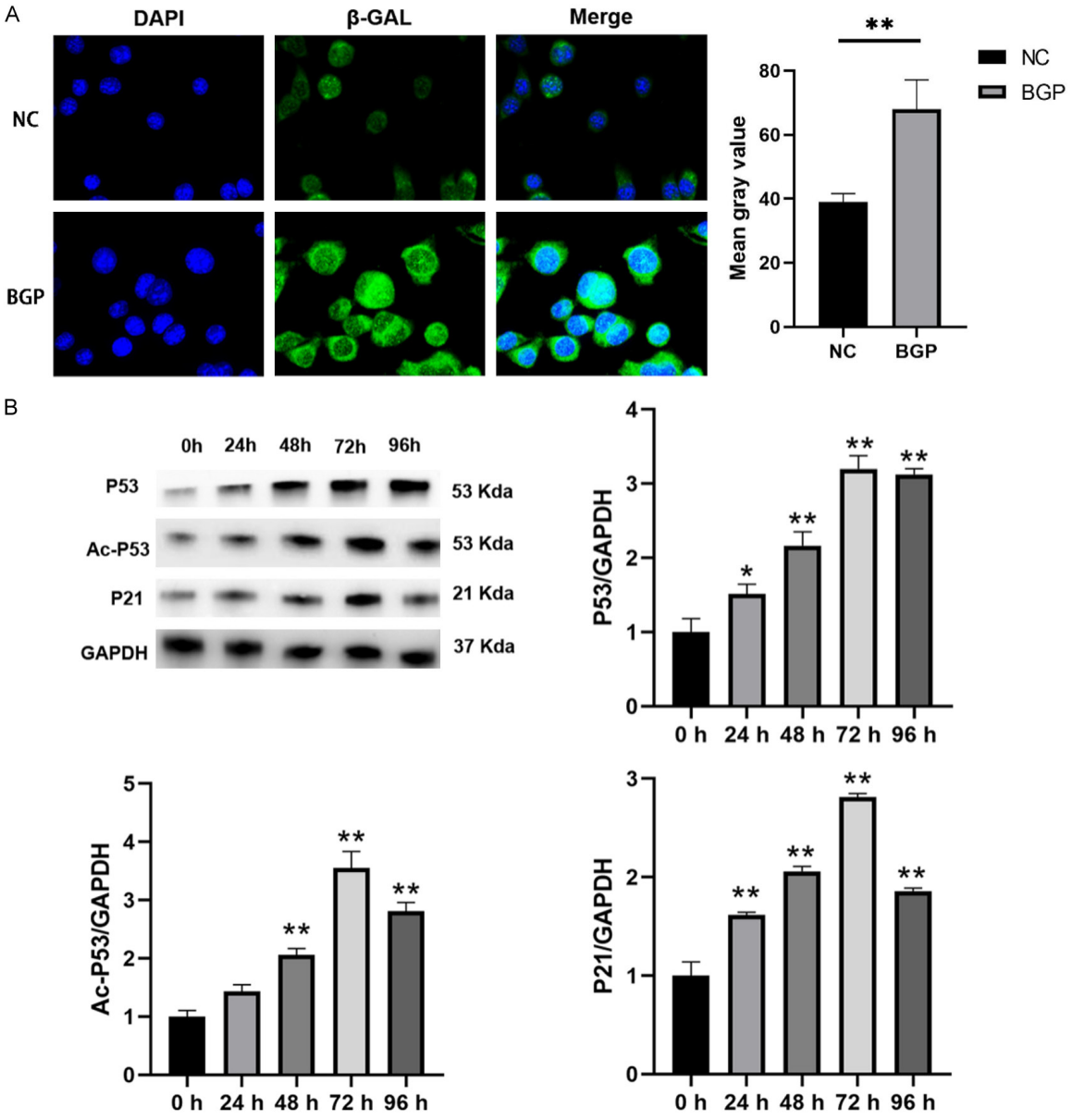

Figure 4. High phosphates induced senescence of C2C12 cells. A. Laser confocal detection of β-GAL expression in C2C12 cells. B. Protein expression of AC-P53, P53 and P21 in C2C12 cells at 0 h, 24 h, 48 h, 72 h, 96 h of high phosphates exposure. *P < 0.05, **P < 0.01, compare with the 0 h, n = 3.

tive or feedback response to phosphate exposure.

High phosphate exposure induced senescence in C2C12 cells by inhibiting SIRT3

To further elucidate the role of SIRT3 in phosphate-induced cellular senescence, C2C12 myoblasts were treated with 2-APQC, a selective SIRT3 activator. Western blot analysis revealed that BGP treatment significantly upregulated the expression of senescence markers P53 and P62, while reducing the ratio of autophagy-related proteins LC3BII/LC3BI (**Figure 5A-D**). P53 is a well-established marker of cellular senescence and stress response, while P62 is an autophagic receptor that accumulates when autophagy is suppressed. These results suggest that high phosphate levels induce cellular senescence by impairing autophagy, a process crucial for maintaining cellular homeostasis and preventing the accumulation of damaged proteins and organelles.

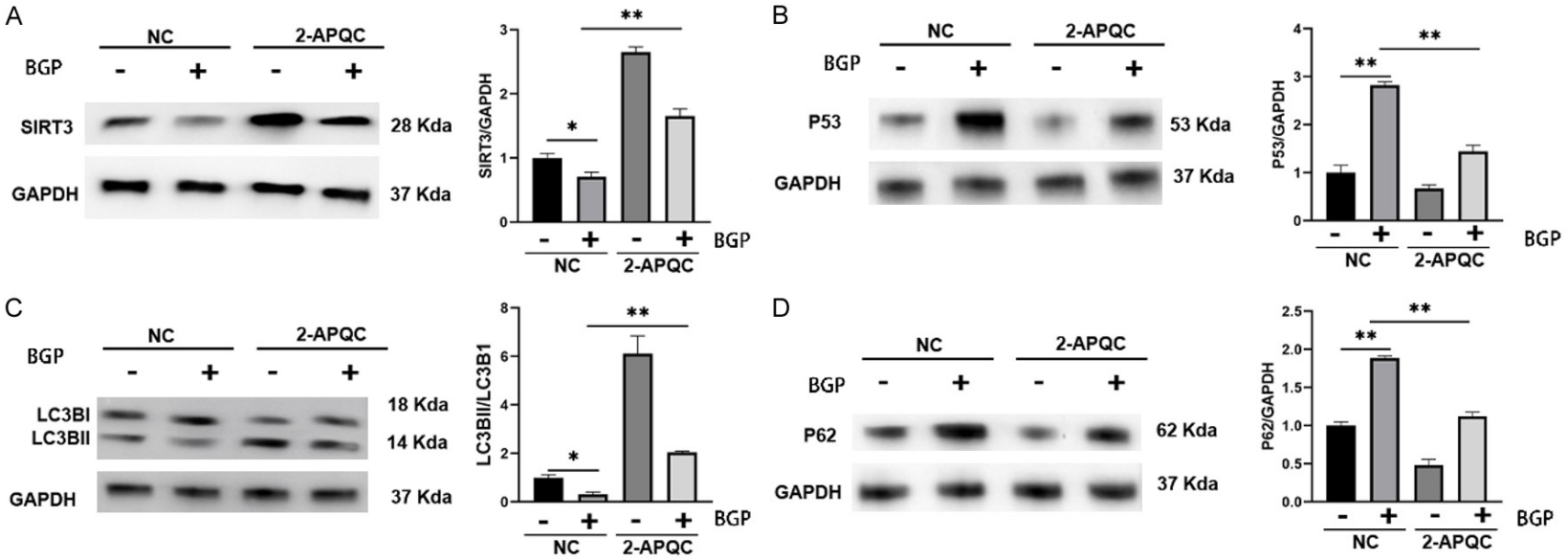

Figure 5. Effect of SIRT3 activation on cellular senescence. Protein expression of SIRT3 (A), P53 (B), LC3BII/LC3BI (C) and P62 (D) in C2C12 cells was assessed by WB. *P < 0.05, **P < 0.01, n = 3.

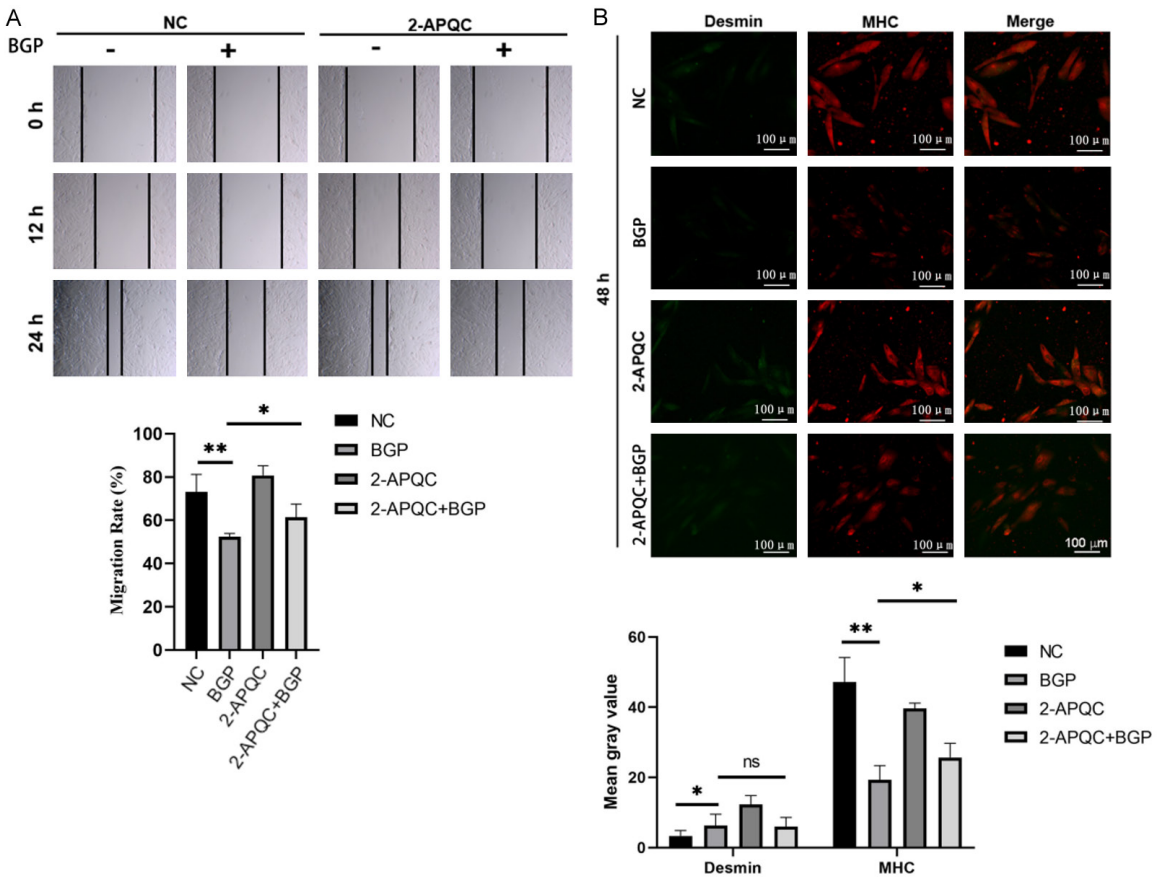

Figure 6. Effect of activation of SIRT3 on the behavior of hyper-phosphate C2C12 cells. A. Cell scratch assay to detect the migratory capacity of cells. B. Immunofluorescence analysis of myosin heavy chain (MHC, red) and desmin (green) expression in C2C12 cells. The results are expressed as the mean ± SD. *P < 0.05, **P < 0.01, n = 3.

Upon co-treatment with 2-APQC, the expression levels of P53 and P62 were markedly reduced, and the LC3B-II/LC3B-I ratio was significantly restored to levels comparable to those in control cells. These results suggest that the activation of SIRT3 by 2-APQC not only mitigates phosphate-induced cellular senescence but also restores autophagic function, which is critical for the clearance of damaged cellular components and the maintenance of cellular integrity.

Activation of SIRT3 ameliorated high phosphates-induced impairments in C2C12 cell migration and myogenic differentiation

High phosphate treatment significantly impaired the migratory ability of C2C12 cells, as evidenced by a marked reduction in wound closure rate compared to control cells (**Figure 6A**). This suggests that elevated phosphate levels compromise cellular processes essential for wound healing and tissue regeneration. However, co-treatment with the SIRT3 activator 2-APQC partially restored the migration potential of C2C12 cells, indicating that SIRT3 plays a protective role in maintaining cellular motility under phosphate stress.

To further evaluate the effects of high phosphate exposure on myogenic differentiation assay, the expression of myosin heavy chain (MHC), a key marker of myogenic differentiation, was examined. The results revealed that high phosphate exposure significantly suppressed MHC expression, indicating impaired myotube formation and differentiation capacity. MHC is essential for the structural integrity and contractile function of muscle fibers; thus, its downregulation reflects compromised muscle development. Interestingly, treatment with 2-APQC significantly increased MHC expression in phosphate-treated cells, alongside a notable co-localization of MHC with desmin, a pro-

tein that stabilizes muscle fibers during differentiation (**Figure 6B**). This restoration of MHC expression and its co-localization with desmin supports the conclusion that SIRT3 activation can effectively counteract the detrimental effects of high salt-phosphates on muscle cell differentiation.

Collectively, these findings demonstrate that high salt-phosphates disrupt both the migration and differentiation of C2C12 cells by downregulating SIRT3 expression. Activation of SIRT3 via 2-APQC can partially rescue these effects, thereby promoting muscle cell regeneration and function.

Discussion

Aging is an inevitable biological process characterized by the progressive deterioration of muscle strength, which adversely affects the quality of life and increases mortality risk among the elderly. Despite extensive research, the molecular mechanisms of muscle aging remain inadequately explored. Emerging evidence suggests that elevated phosphate levels may contribute to cellular senescence. This study aims to elucidate the effects of high phosphate levels on muscle senescence, emphasizing their role in muscle mass, functional capacity, and expression of senescence-associated markers.

To examine the connection between increased phosphate levels and skeletal muscle degeneration, we examined physiological markers in both young and aged mice cohorts. Aged mice exhibited significantly elevated serum sodium levels, consistent with previous findings [21, 22]. Alongside elevated sodium, aged mice exhibited pronounced muscle aging characteristics, such as reduced muscle mass and diminished grip strength - key indicators of age-related muscular decline observed in humans [23]. At the molecular level, expression of key senescence-associated genes, P16, P21, and P53, was significantly upregulated in aged muscle tissues, signifying enhanced cellular senescence [24]. These observations collectively underscore the association between increased sodium and phosphate levels with muscle senescence in mice. Comparable results have been reported in Drosophila models, where phosphate overload induced myogenic inhibition and muscle atrophy via the NAD+/dSir2/dFOXO signaling pathway and elevated oxidative stress [10]. Taken together, our findings reaffirm the critical relationship between high phosphate environments and muscle aging.

Delving into cellular mechanisms, we modeled phosphate-induced senescence in vitro by treating C2C12 cells with 20 mM BGP. A significant increase in SA-β-GAL activity, an established hallmark of cellular aging, was observed, implying that elevated extracellular phosphate directly induces myogenic senescence through upregulation of aging-related genes. Furthermore, high phosphate conditions markedly impaired C2C12 cell migration, a vital process for myogenic repair and skeletal muscle regeneration post-injury [25]. These results highlight the disruptive effects of high phosphate conditions on muscle development, particularly through the inhibition of critical processes like cell migration and differentiation, which are vital for maintaining muscle integrity and function.

SIRT3 has emerged as a hot focus in the study of aging and muscle senescence due to its notable links to enhanced human longevity. In our animal models, SIRT3 expression was significantly reduced in aged muscle tissues, consistent with previous studies associating SIRT3 deficiency with impaired metabolic adaptability and protein stability [26]. To investigate its therapeutic potential, we employed the SIRT3 agonist 2-APQC, which attenuated the expression of phosphate-induced senescence markers, suggesting a protective and anti-aging role for SIRT3 in muscle tissues. Beyond its role in the stress response, SIRT3 is also a critical regulator of autophagy, a cellular recycling process essential for muscle maintenance and metabolic homeostasis [27]. Autophagic dysfunction leads to the accumulation of damaged organelles, misfolded proteins, and increased reactive oxygen species (ROS), fueling muscle atrophy, mitochondrial impairment, and apoptotic pathways [28, 29]. In our study, phosphate treatment suppressed autophagic activity in C2C12 cells, as evidenced by a reduced LC3BII/LC3BI ratio. Conversely, 2-APQC treatment enhanced autophagic activity, further substantiating SIRT3's pivotal role in mitigating phosphate-induced muscle damage.

While this study provides valuable insights, several limitations should be acknowledged. First, the absence of clinical samples limits the trans-

lational relevance and precludes direct validation of the association between elevated phosphate levels, SIRT3 expression, and muscle aging in humans. Second, other critical aging mechanisms, such as mitochondrial dysfunction, telomerase activity, and apoptosis signaling, were not thoroughly investigated. Future research must address these aspects to present a holistic understanding of muscle aging.

In summary, our study establishes a connection between increased serum sodium levels and diminished SIRT3 expression with muscle senescence in mice. At the cellular level, elevated phosphate concentrations promote senescence markers in C2C12 cells, impair myogenesis, and exacerbate muscle dysfunction. Notably, activation of SIRT3 effectively alleviates phosphate-induced aging and dysfunction, underscoring its therapeutic potential in combating muscle senescence. Strategies targeting SIRT3 modulation in combination with restricted phosphate intake may represent a novel intervention in sarcopenia treatment.

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Disclosure of conflict of interest

None.

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