High phosphate levels promote muscle atrophy via myostatin expression in differentiated L6 myotubes

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Abstract

Background: Sarcopenia is the age-induced, progressive loss of skeletal muscle mass and function. This phenomenon is observed in patients with chronic kidney disease (CKD). However, the intracellular mechanism underlying the progressive sarcopenia in CKD has not been completely elucidated. Although hyperphosphatemia contributes to cellular senescence, it is unclear whether this condition induces skeletal muscle atrophy. The aim of this study was to determine the effect of hyperphosphatemia on skeletal muscle.

Methods: Differentiated rat myoblast cells (L6) were exposed to normal (0.9 mM; CON), medium (2.5 mM), and high (3.8mM; HPi) phosphate conditions for 10 days. We measured the protein levels of myosin heavy chain (MHC) and myostatin and determined the ratio of phosphorylated p70S6K and cleaved caspase-3 by western blot. The expression levels of the myogenic transcriptional regulators MyoD and myogenin were measured by qPCR.

Results: The levels of MHC were gradually downregulated depending on the phosphate concentration. Myostatin in HPi was about 20 times higher than in CON ($P<0.001$). In myotubes cultured in HPi, protein synthesis was significantly lower, and degradation was significantly higher than in CON ($P<0.01$). The mRNA expression of MyoD in HPi was significantly lower than in CON.

Conclusions: This study showed that hyperphosphatemia strongly induced muscle atrophy with the accumulation of myostatin. This mechanism might include the downregulation of protein synthesis, upregulation of proteolysis, and attenuated muscle regeneration.

Introduction

Extending the healthy life span and improving the quality of life (QOL) of patients are important in a super-aging society, such as Japan. Sarcopenia, defined as the gradual decline in skeletal muscle mass and strength with aging, may be associated with these important matters. In chronic kidney disease (CKD), mortality is related to the loss of muscle mass [1] and decreases in body mass index (BMI) [2, 3], so the maintenance of muscle mass is essential in both the elderly and CKD patients.

The main features of CKD include the retention of phosphorus (hyperphosphatemia), low-serum calcium (hypocalcemia), and increased fibroblastic growth factor 23 (FGF23). [4, 5] Skeletal muscle is affected by CKD, which causes structural and functional abnormalities known as uremic myopathy [6]. In fact, sarcopenia is prevalent and particularly prominent among uremic patients with end-stage renal disease requiring dialysis [7]. It is extremely difficult to elucidate the mechanisms by which CKD negatively impacts skeletal muscle because they are likely multifaceted and complex, resulting from alterations in muscle perfusion, substrate delivery, catabolic acidosis, corticosteroids, proinflammatory cytokines, and decreased physical activity. Moreover, patients with CKD often have malnutrition that is partially caused by the spontaneous restriction of their dietary protein, which may also affect the degeneration/regeneration balance of skeletal muscle. By using rodent myoblast cell lines, several

researchers have reported that uremic toxins, such as indoxyl sulfate, [8–10] have an atrophic effect on skeletal muscle. However, the intracellular mechanism underlying the progressive sarcopenia in CKD has not been completely elucidated yet. In particular, it is not clear whether a high-phosphate condition, such as hyperphosphatemia, which occurs in CKD and strongly contributes to the cellular senescence in aging, directly has an atrophic effect on skeletal muscle.

Myostatin, a transforming growth factor-beta (TGF-β) superfamily member, is a secreted growth factor that is a well-established negative regulator of muscle growth. In an animal model study, myostatin was increased in both the serum and skeletal muscle in the uremic condition [11]. Therefore, we investigated whether hyperphosphatemia can lead to muscle atrophy with increased myostatin levels using differentiated L6 myoblasts.

Materials And Methods

Cell culture. Rat L6 myoblasts were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (JCRB9081, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan). L6 myoblasts were seeded in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum (Equitech-Bio, Inc., TX, USA), 1% penicillin-streptomycin solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and incubated at 37 °C and 5% CO_2. After the cells reached 80–90% confluency, the myoblasts were differentiated into myotubes with DMEM supplemented with 2% horse serum for 5–7 days. To generate medium- or high-phosphate conditions, 0.1 mol/L NaH_2PO_4 and Na_2HPO_4 were added in equal amounts to the DMEM supplemented with 2% horse serum to obtain a final PO_4^{3-} concentration of 2.45 mmol/L (MPi) or 3.8 mmol/L (HPi).

Following the completion of the 10-day culture period with 2% horse serum-supplemented DMEM (CON), MPi, or HPi, the myotubes were washed twice in calcium- and magnesium-free Hanks' balanced salt solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and used for the following measurements.

Western blot analysis. Myotubes in 6-well plates were lysed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Tokyo, Japan) and sonicated to extract all proteins. The Western blot procedure was previously described [12]. Briefly, protein concentrations were quantified using a commercial reagent (Pierce BCA Protein Assay Kit; Pierce, IL, USA) and normalized to 1 μg/μL for all samples. A total of 5 or 15 μg of protein was loaded into each lane of a 5–20% precast e-PAGEL (ATTO Co., Tokyo, Japan). The gel was transferred to a polyvinylidene difluoride (PVDF) membrane using Ezblot (AE-1460, ATTO, Tokyo, Japan) reagent and a semi-dry blotting unit (WSE-4110 PoweredBLOT One, ATTO, Tokyo, Japan) according to the manufacturers' instructions. After transfer, the blots were blocked with a commercial blocking reagent (Can Get Signal PVDF Blocking Reagent; Toyobo, Osaka, Japan) for 1 h at room temperature. After washing in Tris-buffered saline (50 mM Tris, 150 mM NaCl; pH 7.6) containing 0.1% Tween 20 (TBST), the blots were incubated overnight at 4 °C with primary antibodies diluted in an

immunoreaction-enhancer solution (Can Get Signal Solution 1; Toyobo). The primary antibodies used in this study were rabbit polyclonal anti-GAPDH (sc-25778; Santa Cruz Biotechnology, CA, USA; diluted 1:1500), rabbit polyclonal anti-caspase-3 (#9662S, Cell Signaling Technology, MA, USA; diluted 1:1000), rabbit polyclonal anti-cleaved caspase-3 (#9661S, Cell Signaling Technology, MA, USA; diluted 1:1000), mouse monoclonal anti-myostatin (GDF-8/11(A-1), sc-398333; Santa Cruz Biotechnology, CA, USA; diluted 1:1000), mouse monoclonal anti-p70 S6 kinase (sc-8418; Santa Cruz Biotechnology, CA, USA; diluted 1:1000), mouse monoclonal anti-p-p70 S6 kinase (sc-8416; Santa Cruz Biotechnology, CA, USA; diluted 1:1000), and mouse monoclonal anti-human myosin heavy chain (MHC, MAB4470; R&D Systems, MN, USA; diluted 1:2000). The membrane was washed in TBST and then reacted with a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (ab16284; Abcam, Cambridge, MA, USA) diluted in an immunoreaction-enhancer solution (1:5000 in Can Get Signal Solution 2; Toyobo, Osaka, Japan) for 1 h at room temperature. The membrane was washed in TBST and processed using an enhanced chemiluminescence reagent (ECL Prime Western Blotting Detection Reagent; GE Healthcare Japan, Tokyo, Japan). The ECL signals on the immunoblots were detected using the WSE-6100 LuminoGraph (ATTO, Tokyo, Japan) and quantified using ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA).

RNA isolation and RT-qPCR analysis. Total RNA was extracted from L6 myotubes using TRIzol reagent (Thermo Fisher Scientific, Inc., Tokyo, Japan) according to the manufacturers' instruction. To quantify mRNA expression levels, the following primers were used: *Myod1* (Fw; CGGGACACAGACTTGCTAGG, Rv; GTGAGTCGAAACACGGATCA), *Myog* (Fw; TCGAGGCTCTGAAGAGAAGC, Rv; AGGCGCTCAATGTACTGGAT), and 18S rRNA (Fw; AAACGGCTACCACATCCAAG, Rv; CGAAGAGCCCGGTATTGTTA). cDNA was synthesized with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc., Tokyo, Japan). qPCR was performed using the Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Inc., Tokyo, Japan) and analyzed with the 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc., Tokyo, Japan). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method to normalize target gene mRNA to 18S rRNA.

Statistical analysis. All data are presented as the mean ± s.d. The statistical significance among three phosphate conditions (Fig. 1) or between two unpaired groups (Fig. 2–4) were analyzed by one-way ANOVA or a two-tailed Student's t-test, respectively. $P < 0.05$ was considered to be statistically significant.

Results

MHC, a main component of myotubes, was significantly lower in both phosphate-supplemented groups (MPi and HPi) compared with in CON ($p < 0.001$) after the 10-day culture period, whereas MHC in HPi was markedly lower than in MPi ($p < 0.001$) (Fig. 1). In addition, there was a dose-dependent relationship between MHC and the amount of phosphate. Thus, our results show that a high-phosphate condition can downregulate skeletal muscle, indicating sarcopenia. The protein expression of myostatin, a negative regulator of skeletal muscle, in HPi was about 20 times higher than in CON (Fig. 2).

Figure 3 shows both indexes of muscle synthesis (anabolic) and degradation (catabolic) after 10 days of culture. The expression of the phosphorylated form of p70S6K, a critical enzyme of the mTOR protein synthesis pathway, was significantly downregulated in HPi compared with in CON. Meanwhile, the active form of caspase-3, which indicates activation of apoptosis, was significantly higher in HPi than in CON.

We analyzed the mRNA expression of the myogenic transcriptional regulators MyoD and myogenin by RT-qPCR in L6 myotubes cultured for 3 and 7 days. There were no significant differences in the mRNA expression of these proteins in CON and HPi after 3 days of culture (Fig. 4A). Results showed a slight decrease in myogenin ($P = 0.07$) and a significant decrease in MyoD ($P = 0.049$) mRNA expression in HPi compared with in CON after 7 days of culture (Fig. 4B).

Discussion

This study showed that high phosphate levels directly contributed to the loss of skeletal muscle with an increase in myostatin. The phenomenon that low phosphate conditions (hypophosphatemia) could lead to myopathy [13] has been well known since the 1970s. In addition, the previous animal study demonstrated that the decrease in muscle weakness in hypophosphatemia was caused by reduced muscle ATP synthesis [14]. Although the muscle physiology involved in hypophosphatemia has been studied for a long time, the effects of hyperphosphatemia on the homeostasis of skeletal muscle have not been fully elucidated. Recently, a human study by Chen et al. [15] demonstrated an inverse association between serum phosphate levels and muscle strength. To the best of our knowledge, there is only one *in vitro* study that investigated the effect of high-phosphate conditions in mouse C_2C_{12} cells [16]. However, they examined how high phosphate levels affected the differentiation of myoblasts to myotubes, indicating that satellite cells differentiate into myotubes during the process of muscle regeneration. Furthermore, they used 10 mM beta glycerophosphate to generate the high-phosphate condition, which is an extremely high pharmacological dose. Thus, the present study is the first to demonstrate the atrophic effects of a high-phosphate condition on myotubes.

p70S6K is a serine/threonine kinase regulated by the Akt/mTOR pathway, which plays a role in protein synthesis. In addition, myostatin reduces Akt/mTOR/p70S6K signaling [17]. Although we did not measure the activities of these pathways, an increase in myostatin by culturing in high-phosphate conditions in our study might have contributed to the downregulation of MHC and subsequent increase in muscle atrophy. The process of proteolysis includes both the ubiquitin-proteasome system (UPS) and the activation of caspase-3. Because catabolic conditions, such as a CKD, cause muscle atrophy, it has been suggested that activating caspase-3 is essential to cleave actomyosin and break down the complex structure of myofibrils, which cannot be degraded by the UPS [18]. Thus, we thought that the increase in the activated form of caspase-3 indicated protein degradation in the high-phosphate condition. This activated caspase-3 can be led by downregulating PI3K/Akt signaling, which is downstream of Smad 2/3 signaling mediated by myostatin. Therefore, the increased myostatin levels by culturing in a high-phosphate condition in this study might have activated protein degradation via activation of the caspase-3 pathway. Moreover, muscle atrophy results from a change in the ratio of protein synthesis to

degradation, and a net loss of muscle protein could result from either increased degradation, decreased synthesis, or a combination of the two. In this context, the high-phosphate conditions observed in CKD could induce significant muscle atrophy.

In this study, the mRNA levels of muscle regulatory factors were decreased in the high-phosphate condition, which led to the accumulation of myostatin in myotubes. Myostatin also suppresses the gene expression of several myoregulatory factors, such as MyoD and myogenin, via the phosphorylation of Smad 3 [19], suggesting that the ability of regeneration in skeletal muscle is attenuated. Although we did not directly measure muscle regeneration, the downregulation of MyoD induced by an increase in myostatin in the present study might have attenuated muscle regeneration and caused lower MHC expression.

In conclusion, hyperphosphatemia caused muscle atrophy that was induced by an accumulation of myostatin. This mechanism might include the downregulation of protein synthesis, upregulation of proteolysis, and attenuation of muscle regeneration.

Declarations

Ethics approval

Not applicable

Competing interests

T. Shigematsu. has received lecture fees from Chugai Pharmaceutical Co., Ltd.; lecture and consultancy fees from Kyowa Kirin Co., Ltd.; Grants and consultancy fees from Fuji Yakuhin Co., Ltd.; and Grants, lecture and Consultancy fees from Ono Pharmaceutical Co., Ltd. The authors declare that they have no other relevant financial interests.

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Authors' contributions

T.S. and M.O. designed the study, performed data analysis, and wrote the first draft of the manuscript. T.S., K.K., and M.Y. performed data acquisition. T.M, S.N., and T.S. supervised or mentored the study. All authors contributed to the interpretation of the data and critically revised the final draft of the manuscript.

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Figures

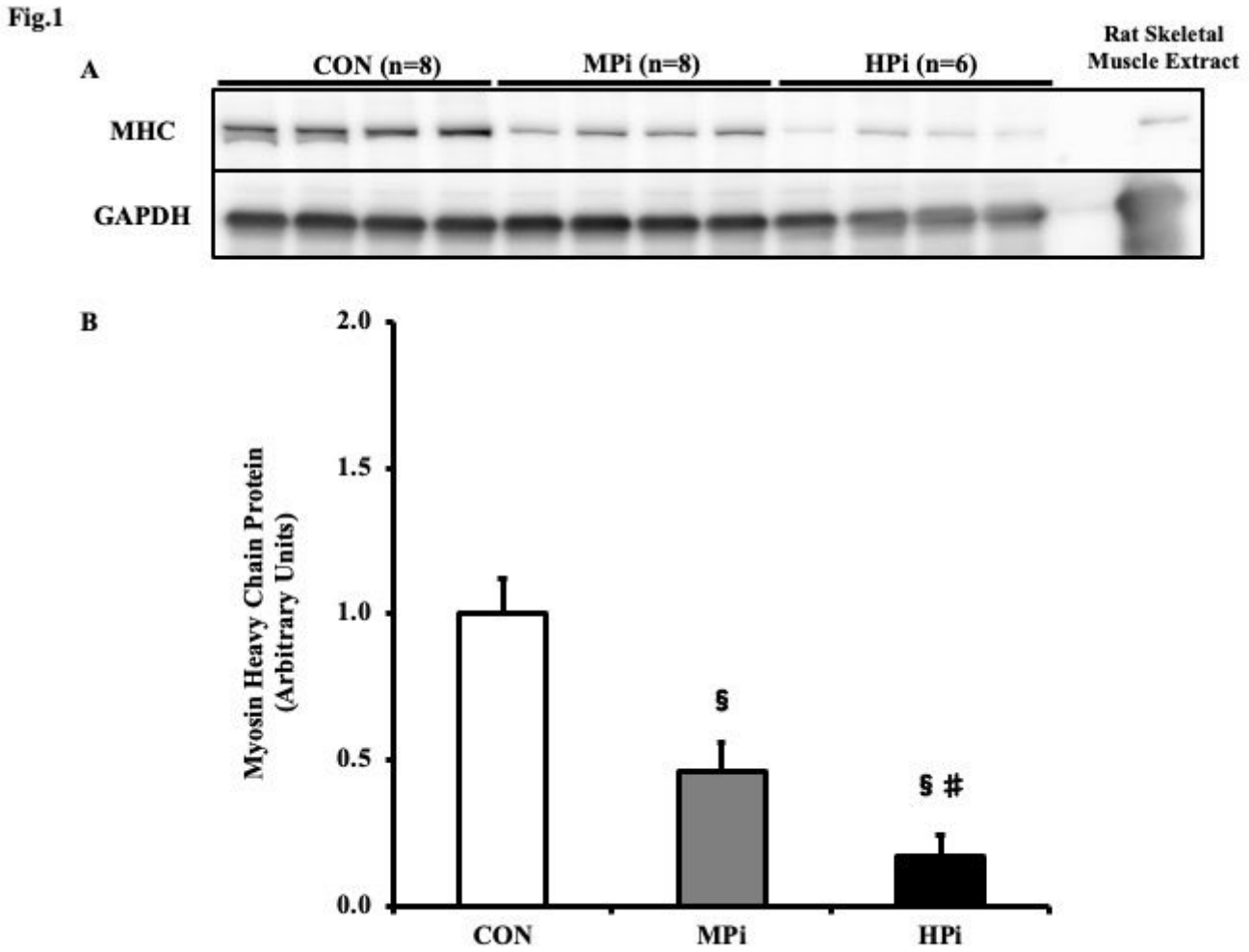

Figure 1

Relative expression level of MHC in L6 myotubes cultured for 10 days in various phosphate concentrations. A: Representative western blot bands of each condition. B: The bar graph represents the relative densitometric analysis of each band normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels. These densitometric data were obtained from two separate membranes blotted by the samples derived from the same experiment and that blots were processed in parallel. § indicates statistically significant differences compared with CON (P<0.001). # indicates statistically significant differences compared with MPi (P<0.001). CON, control medium; MPi, medium-phosphate medium; HPi, high-phosphate medium.

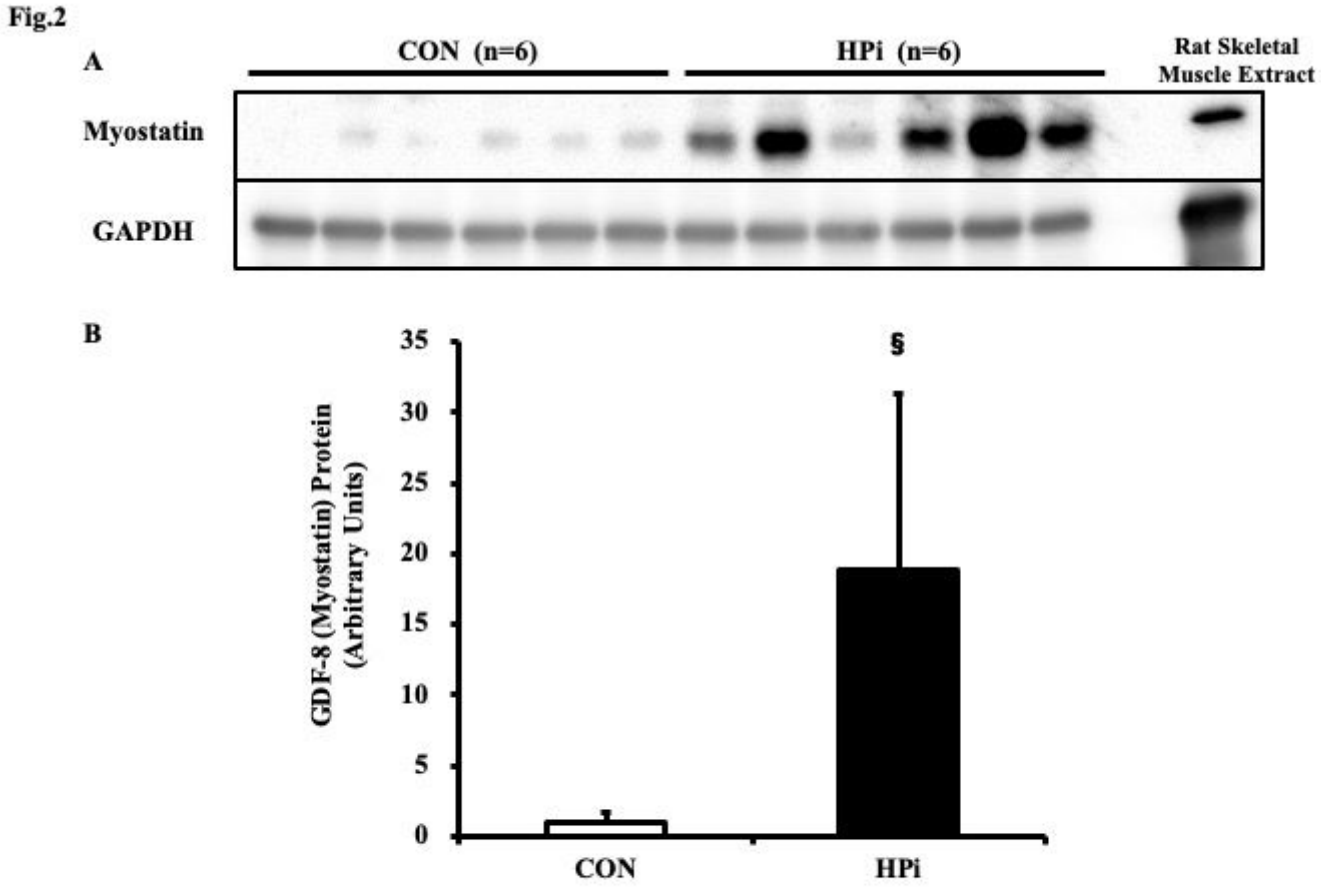

Figure 2

Relative expression level of myostatin in L6 myotubes cultured for 10 days. A: Western blot bands of each condition. B: The bar graph represents the relative densitometric analysis of each band normalized to GAPDH protein levels. § indicates statistically significant differences compared with CON (P<0.001). CON, control medium; HPi, high-phosphate medium.

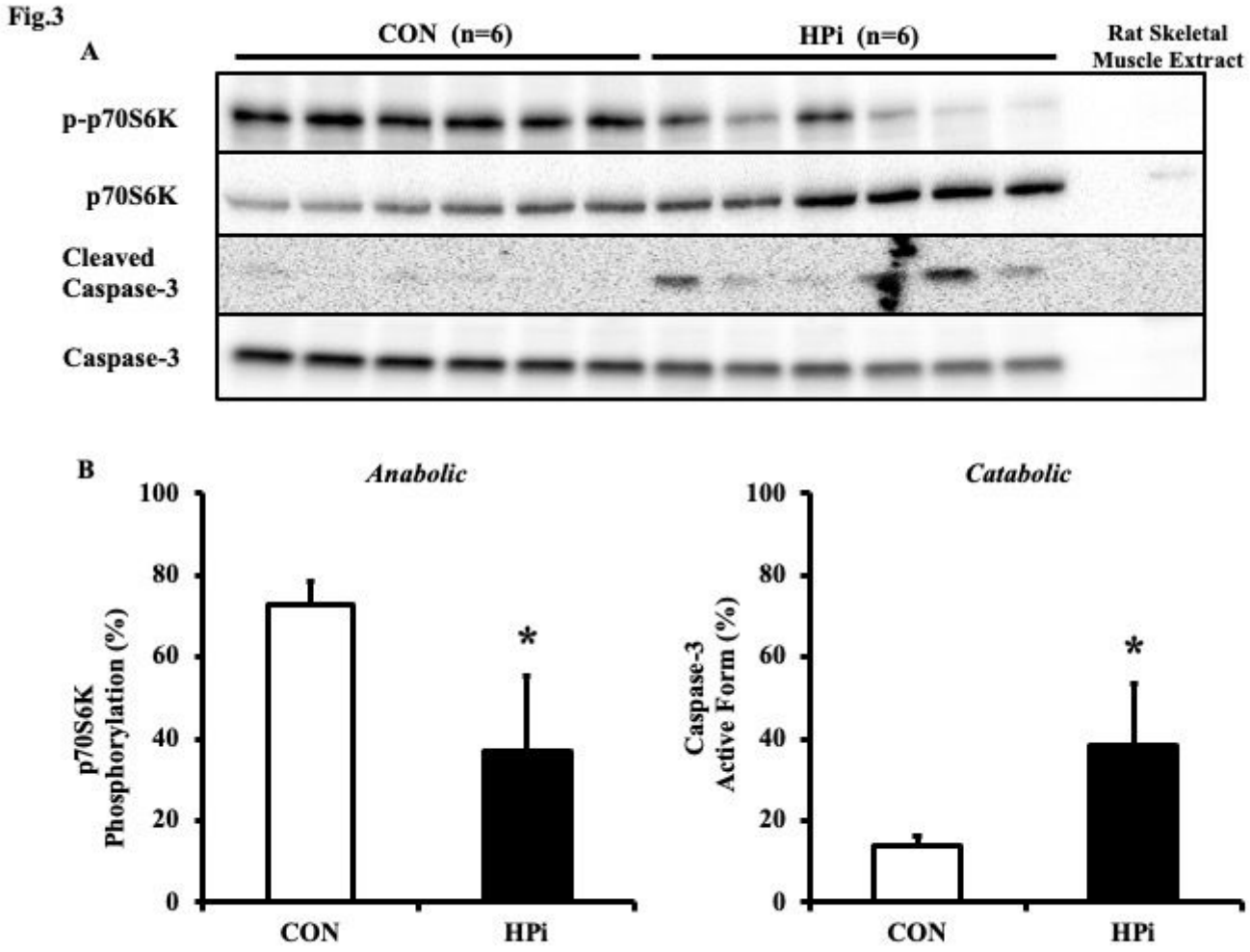

Figure 3

Anabolic and catabolic indexes in L6 myotubes cultured for 10 days in various phosphate concentrations. A: Western blot bands of each protein. B: The anabolic index is indicated by the ratio of phosphorylated p70S6K to total p70S6K protein levels. * indicates statistically significant differences compared with CON ($P<0.01$). C: The catabolic index is indicated by the ratio of cleaved caspase-3 to total caspase-3 protein levels. * indicates statistically significant differences compared with CON ($P<0.01$). CON, control medium; HPi, high-phosphate medium.

Fig.4

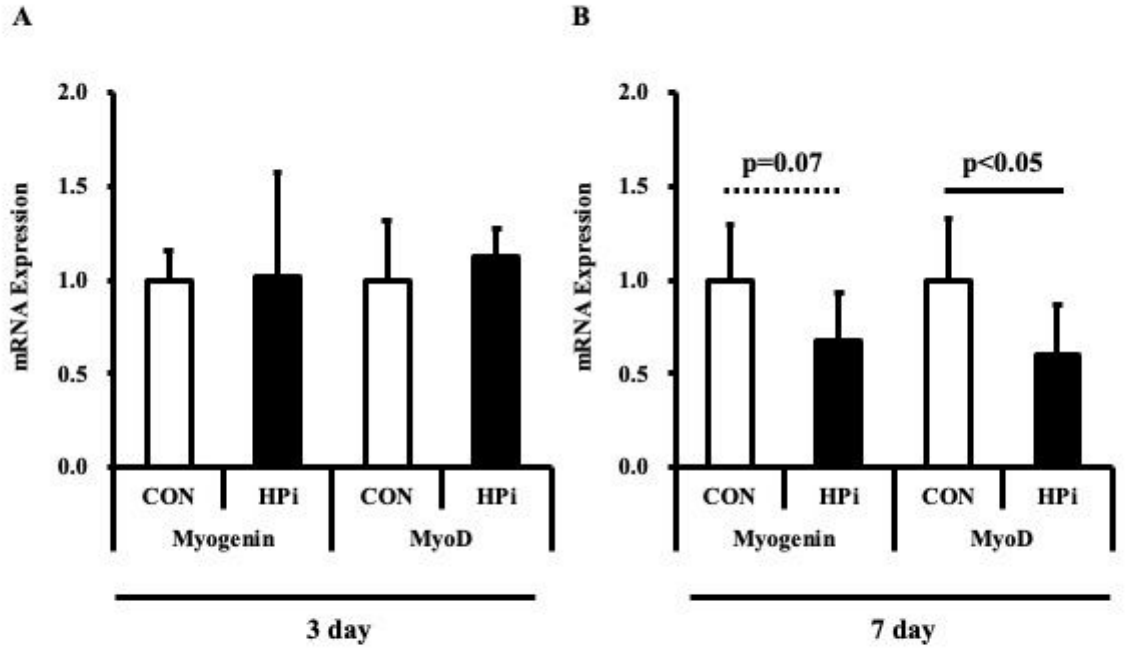

Figure 4

mRNA expression of myogenic transcriptional regulators (MyoD and myogenin) in L6 myotubes cultured for 3 and 7 days (n = 6 in each group). The RT-qPCR data were normalized to 18S rRNA. CON, control medium; HPi, high-phosphate medium.