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Original Article

Reduced expression of carbonic anhydrase III in skeletal muscles could be linked to muscle fatigue: A rat muscle fatigue model

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ABSTRACT

Background: Carbonic anhydrase III (CAIII) is expressed abundantly in slow skeletal muscles, adipocytes, and the liver. It plays a critical role in maintaining intracellular pH, antioxidation, and energy metabolism, which are further involved in fatigue. However, its function and mechanism in maintaining the physiological function of muscles or antifatigue are still ambiguous. We hypothesized that changes of CAIII in skeletal muscles might be related to the occurrence of muscle fatigue.

Method: After establishing a rat soleus muscle fatigue model, we measured the protein expression of the CAIII in muscles. And the muscle intracellular biochemical indices [malondialdehyde (MDA), adenosine triphosphate (ATP), and lactic acid] were also measured using assay kits. After transfected by CAIII-overexpressing and knockdown lentiviral vectors, the rat soleus muscles were induced to fatigue to investigate the effects and possible molecular mechanisms of CAIII in antifatigue.

Results: The expression of CAIII in fatigued soleus muscles was significantly decreased compared with that of the control group ($P < 0.001$). Moreover, the ATP level in the fatigued muscle also significantly decreased, whereas lactic acid and MDA levels were significantly increased ($P < 0.001$). After posttransfection for 21 days, CAIII levels in muscles were significantly reduced in the CAIII-interfering lentivirus group, but increased in the CAIII-overexpressed lentivirus group ($P < 0.001$). In addition, CAIII knockdown muscles showed more reduction of the maximal muscle force and ATP levels and more increase of MDA and lactic acid levels during the fatigue test than the control group, ($P < 0.05$). On the other hand, CAIII-overexpressed muscles showed less reduction of the maximal muscle force and ATP levels and less increase of MDA and lactic acid levels during muscle fatigue than the control group ($P < 0.05$).

Conclusions: Our study showed that soleus muscle fatigue induced by electrical stimulation could result in downregulation of CAIII and ATP levels and accumulation of lactic acid and MDA. Further study showed that CAIII knockdown led to more reduction of the maximal muscle force, whereas CAIII overexpression showed less reduction of the maximal muscle force, which suggested that CAIII levels in muscles might be related to the occurrence of muscle fatigue.

Translational potential: CAIII plays an important role in muscle fatigue. Up-regulating the expression of CAIII might contribute to dissipating fatigue, which would provide a new method to solve the difficulties in eliminating muscular fatigue.

Abbreviations: ATP, adenosine triphosphate; CAIII, carbonic anhydrase III; CAs, carbonic anhydrases; Ct, threshold cycle; MDA, malondialdehyde; NS, saline injection group; PBS, phosphate-buffered saline; PBST, PBS with Tween 20; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; SD, Sprague Dawley; SDS–PAGE gel, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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Introduction

Exercise-induced muscle fatigue is a physiological process, which is generally defined as a reduction in maximal force or power production in response to contractile activity [1]. The mechanism underlying muscle fatigue is exceptionally complex, including coupling, energy metabolism, and changes in the chemical composition of vital elements in the muscles [2,3]. Some of the possible mechanisms postulated are that under strenuous exercise, fatigue is induced in the skeletal muscles owing to accumulation of specific metabolites that bring about changes of intracellular pH, increase the formation of free radicals and reactive oxygen species (ROS), and decrease ATP levels in muscles cells [4–7]. However, the exact underlying mechanisms of fatigue are still unclear. Carbonic anhydrase III (CAIII) is a member of the carbonic anhydrase (CA) family, which is abundantly expressed in the skeletal muscle, the liver, and adipocytes at approximately 10%, 8%, and 24% of the total cytoplasmic proteins, respectively [8,9]. CAIII plays a pivotal role in regulating intracellular pH, maintaining acid–base balance, antioxidation, and energy metabolism, which are involved in the occurrence and development of fatigue [7,9–11].

Moreover, CAIII was found to be suppressed in several diseases that were linked to fatigue and muscle pain such as rheumatoid arthritis and systemic lupus erythematosus [12]. Du et al. [13] found that the levels of CAIII in skeletal muscles of patients with myasthenia gravis were noticeably lower than those in healthy persons. It is well established that the main clinical manifestations of myasthenia gravis are muscle fatigability and amyosthenia. Thus, it is suggested that muscle fatigability and amyosthenia could be associated with deficiency of CAIII in skeletal muscles of patients with myasthenia gravis. In our previous studies, we detected that CAIII protein levels in the soleus muscle of the rats were significantly reduced after acute high-intensity treadmill exercise–induced fatigue. Similarly, we also observed significantly reduced CAIII expression in the gastrocnemius muscles after low-frequency electrical stimulation–induced muscle fatigue [14].

The analysis of the aforementioned studies suggests a plausible correlation between CAIII expression and muscle fatigue. However, its role in maintaining the physiological function of muscles under fatigue is still under scrutiny. Thus, we hypothesized that the occurrence of skeletal muscle fatigue could be related to the downregulation of CAIII expression. To clarify this hypothesis, we established a rat semi-isolated electrically stimulated soleus muscle fatigue model and detected CAIII expression and biochemical indices in the soleus muscles. Furthermore, we constructed CAIII-overexpressing and knockdown lentiviral vectors and then transfected into rat soleus muscles. The transfected soleus muscles were used to observe the effects and possible molecular mechanisms of CAIII in muscle fatigue. This study will investigate the scientific basis for the correlation between changes in muscle CAIII expression and fatigue and lay the foundation for further research on the molecular mechanisms of their interaction.

Materials and methods

Animals

Adult male Sprague Dawley (SD) rats (200–220 g) were purchased from Shanghai Laboratory Animal Centre (Shanghai, China). All animals were maintained at constant temperature (22 ± 2 °C) and humidity, were fed standard rat feed ad libitum, had free access to water, and were under a 12-h light–dark cycle. Animal surgery was performed under sterile conditions and anaesthetized using sodium pentobarbital (40 mg/kg). The Ethics Committee of Huashan Hospital, Fudan University, approved the experimental design (2018-JS075), and all animal experiments strictly complied with the regulations of the standards of the China Animal Health Committee.

Semi-isolated electrical stimulation skeletal muscle fatigue model

A modified in situ electrically stimulated skeletal muscle fatigue model was established according to previously reported studies [15–18]. Soleus muscles from the rats were used for the experiments. After being anaesthetized using sodium pentobarbital (40 mg/kg), the SD rats were placed on a temperature-controlled platform and under an infrared heat lamp to maintain body temperature during the complete experimental procedure. Hair was trimmed from the posterior aspect of the lower leg, and a midline longitudinal incision was made on the posterior aspect to dissect the gastrocnemius muscle and expose the soleus muscle. The proximal end of the soleus muscle was preserved, whereas the distal tendon of the soleus muscle was surgically exposed, dissected, and attached to the force transducer (SMUP-E biological signal processing system, Chengdu Equipment Factory, Chengdu,China) using a 4/0 silk suture to hold the tendon tightly. The muscle was stimulated by applying electrical pulses through parallel platinum field electrodes placed on the proximal portion (motor point) of the soleus muscle. The electrodes were not in contact with the nerve branch or the fibre itself but stimulated the muscle via the surrounding fluid. MFLab 200 software (Fudan University, Shanghai, China) was used to record a spontaneous contraction. Warm saline was continuously added to the muscles until the end of the electrical stimulation. Constant temperature and wet conditions were maintained throughout the experiment. The muscles were initially adjusted to the optimum length (Lo). The electric stimulation fatigue test was performed with 1 tetanic contraction at 10 Hz every 5 s and a single train duration of 0.5 s. To investigate the expressions of CAIII, the rats were randomly divided into 4 groups: the control group (sham group in which all procedures were performed but without the electrical stimulation) and three electrical stimulation groups with different duration of stimulus 5-min, 10-min, and 20-min group, respectively (each group, n = 5). The graphic representation of electrical stimulation of the soleus muscle fatigue model is shown in Fig. 1.

Construction of lentiviral RNA interference and the recombinant lentiviral vector

Based on our earlier studies, optimal CAIII RNA interference (RNAi) oligonucleotide target sequences were synthesized by GeneChem (Shanghai, China) and cloned into the GV248 plasmid for knockdown of CAIII as described [19,20]. Similarly, for overexpression of CAIII, the DNA segment of CAIII was cloned into the GV358 lentiviral vector as described [21,22]. Based on the findings from these studies, we used lentiviral titres for GV248 and GV358 at 5E+8 and 2E+8 transducing U/ml, respectively.

Lentiviral vector skeletal muscle transfection and its effects on muscle fatigue

To determine the optimal time point of lentivirus transfection in the skeletal muscle, the SD rats were anaesthetized, and 20 μl of empty lentivirus vector–containing green fluorescent protein (GFP) was injected into the rat soleus muscle in situ on Day 0. After transfection on Days 7, 14, and 21, the animals were sacrificed and the soleus muscle was dissected and observed under the fluorescence microscope. The optimal fluorescence signal was observed at 21 days after injection (shown in Fig. 2), suggesting effective transfection of the skeletal muscles. To investigate the influence of CAIII gene silencing or overexpression on muscle fatigue, the SD rats were randomly divided into 4 groups: physiological saline injection group (control), empty virus injection group (C-Virus), interference virus injection group (RNAi), and overexpression virus injection group (GV358-CAIII) (each group, n = 5). After 21 days after transfection, the rat soleus muscle electric stimulation fatigue test was performed as mentioned previously. The stimulation signals were recorded synchronously by using a biological signal processing system. At the end of the electric stimulation fatigue test, the rat soleus muscles

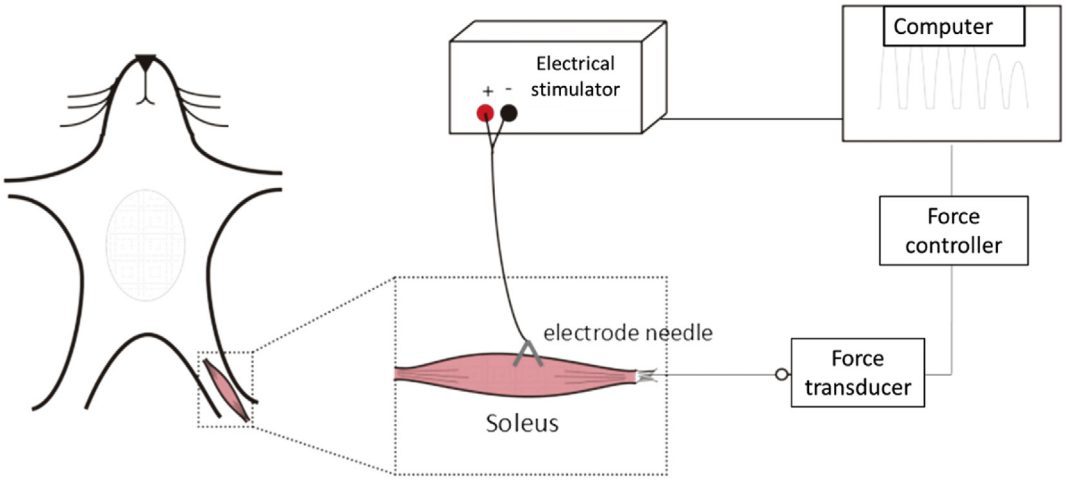

Figure 1. Schematic diagram of semi-isolated electrical stimulation soleus muscle fatigue model: the SMUP-E biological signal processing system was used to record the stimulation signal in real time. The soleus muscle was first adjusted to the optimum length (Lo). Then, electric stimulation fatigue protocol was performed with 1 tetanic contraction at 10 Hz every 5 s and a single train duration of 0.5 s for stimulation duration of 5 min, 10 min, and 20 min, respectively. During muscle electric stimulation, constant temperature and wet condition were maintained by continuously adding warm saline to the muscles.

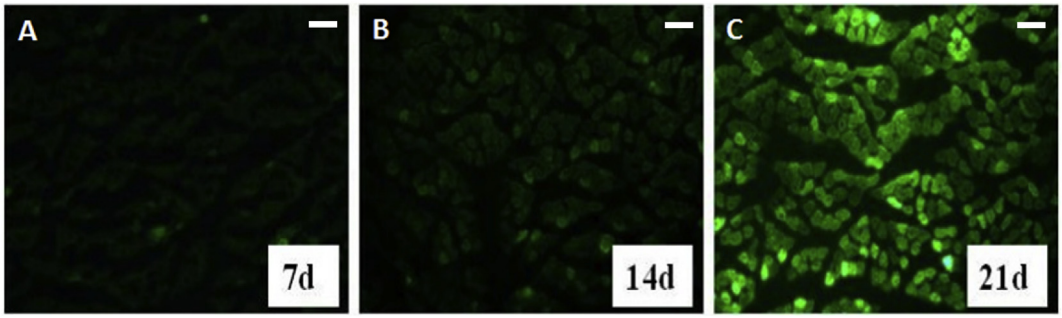

Figure 2. Lentiviral vector transfection of the rat soleus muscle. SD rats were anaesthetized and 20 μl of empty lentivirus vector–containing GFP was injected into the rat soleus muscle. The rats were sacrificed on (A) Day 7, (B) Day 14, and (C) Day 21, and the soleus muscle was harvested and observed under the inverted fluorescence microscope for GFP expression (scale bar = 100 mm). SD = Sprague Dawley; GFP, green fluorescent protein.

were extracted, snap frozen, and stored at 80 °C for subsequent analysis. Finally, the rats were sacrificed by cervical dislocation.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and Western blot analysis

From the harvested and stored soleus muscles, 200 mg of tissues was washed with 0.01 M phosphate-buffered saline (PBS), placed in a 1.5-ml round-bottom Eppendorf tube, cut into pieces, and homogenized with 0.5 ml of radioimmunoprecipitation assay solution (Beyotime Institute of Biotechnology, Jiangsu, China) on an ice homogenizer. Then, the mixture was centrifuged at 12,000 r/min for 20 min at 4 °C, and the supernatant was collected. The protein concentration of the supernatant was determined by the BCA protein quantitative assay (Sangon Biotech Co., Ltd, Shanghai, China). A total of 10 μg protein sample was separated on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The protein bands in the sodium dodecyl sulphate–polyacrylamide gel were visualized by staining with Coomassie blue. The total protein in each lane was quantified using ImageJ software (National Institutes of Health (NIH): Bethesda, MA, USA) for normalizing the amount of sample loading, and then, the proteins were transferred onto the polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), which was blocked with 5% nonfat milk for 2 h. Then, the membrane was washed with PBS containing Tween 20 and incubated overnight at 4 °C with rabbit anti–rat CAIII polyclonal antibody (1:1600; Santa Cruz Biotechnology, Delaware, CA, USA). Then, the membrane was washed three times with PBS containing Tween 20 and incubated with goat anti–rabbit IgG (H + L)-HRP (1:2000; Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. The membrane was enhanced using the Clarity Western ECL system (170–5060; Bio-Rad, Hercules, CA, USA). Proteins of interest were quantified by using the ImageQuant LAS 4000 Mini luminescent image analyzer (GE Healthcare, Milwaukee, WI, USA). β-actin (1:1000; Abcam, Cambridge, MA, USA) serves as an internal control.

Real-time polymerase chain reaction

Total RNA was isolated from the muscles using an RNAprep Pure Hi-Blood Kit according to the manufacturer's protocol (Tiangen BIOTECH Co., Ltd., China). The RNA concentration was determined by calculating the OD260:OD280 ratio. Rapid reverse transcription was performed using the PrimeScript RT Reagent Kit (Perfect Real Time; TaKaRa Biotechnology, Japan). Detection of CAIII mRNA levels was performed using Applied Biosystems' quantitative measurement phase + real-time

Table 1
Primer sequences for real-time PCR.

Amplification target	Primer end	Sequence
CAIII	5′	CTCTGGACCCTACCGACTTC
	3′	CCAACCACAGCAATCCCATC
β-actin	5′	CTGTCCCTGTATGCCTCTG
	3′	ATGTCACGCACGATTTCC

CAIII = carbonic anhydrase III; PCR = polymerase chain reaction.

PCR system (Life Technologies, CA, USA). β-actin serves as an internal reference. All primers used in the experiment were synthesized by Sangon Biotech Co., Ltd. The primer sequences used are shown in Table 1. The threshold cycle (Ct) was calculated using the second-derivative maximum method. The data were analyzed via the delta–delta method. The assays were performed three times using triplicate wells. The final values were expressed as the ratio versus the control.

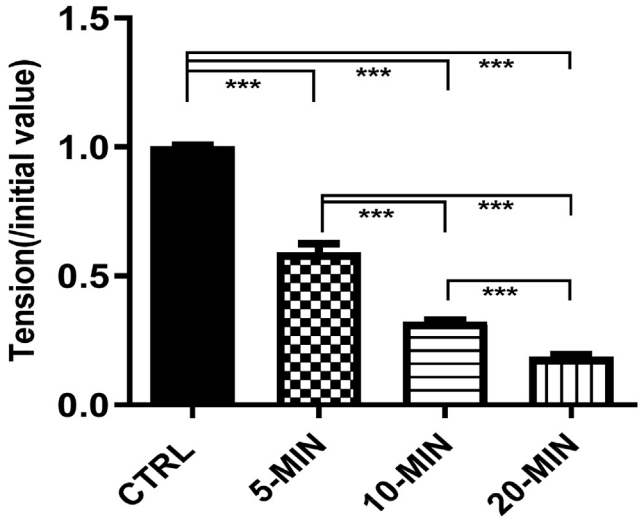

Figure 3. Fatigue characteristics in soleus muscle force after electrical stimulation: Bar chart showing the muscle force of the soleus muscle at various electric stimulation time points 5 min, 10 min, and 20 min, respectively (each group, N = 5). The soleus muscle force decreased gradually with the prolongation of the stimulation time. ***P < 0.001. CTRL = control group.

Detection of the concentration values of MDA, lactate, and ATP in muscle fatigue models

Immediately after the end of electrical stimulation, the soleus muscles (20 mg) were homogenized and centrifuged at 12000 g for 5 min at 4 °C. The extracted supernatant was measured for biochemistry indices, including MDA, ATP (Nanjing Jiancheng Biological Engineering Institute, Jiangsu, China), and lactic acid (Beyotime Institute of Biotechnology), using assay kits according to the manufacturer's instructions.

Image analysis and statistical analysis

All experiments were repeated in triplicates. Data were collected and statistically analyzed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA) and SPSS 19.0 (IBM, Chicago, USA). Experiment values were presented as means ± standard deviation (mean ± standard deviation). Statistical significance was assessed using one-way analysis of variance and the unpaired t test. Differences were considered statistically significant when $p < 0.05$.

Results

Rat semi-isolated electrical stimulation soleus muscle fatigue model

After electrical stimulation, the muscle force of the soleus muscle significantly decreased with the prolongation of the stimulation time. Compared with the control group, there was significant decrease in the muscle force at 5-min (0.59 ± 0.04, P < 0.001) and 10-min (0.32 ± 0.00, P < 0.001) electric stimulation and nearly 80% decrease at 20-min stimulation (0.18 ± 0.01, P < 0.001). The muscle force at 10-min and 20-min stimulation significantly decreased compared with the 5-min stimulation (P < 0.001). In addition, the 20-min stimulation showed a significant decrease in muscle force compared with the 10-min stimulation (P < 0.001) (Fig. 3). It indicated the skeletal muscle electrical stimulation fatigue model was successfully established.

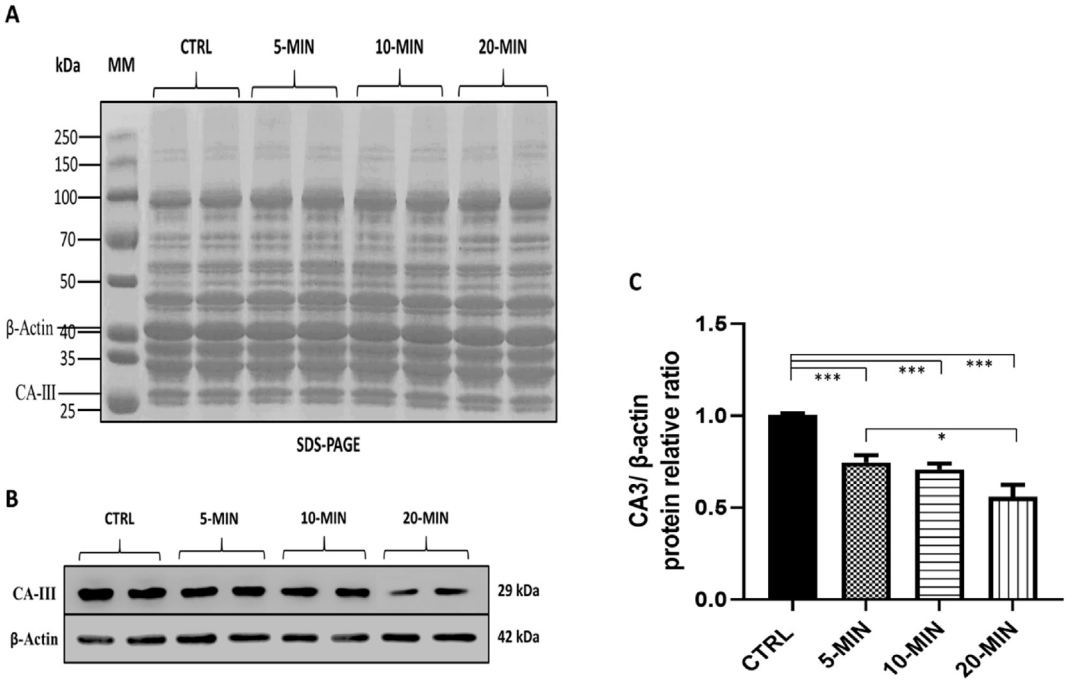

Figure 4. Changes of CAIII protein expression during electrical stimulation of skeletal muscle fatigue: (A) SDS-PAGE gel to normalize the amount of protein for sample loading. (B) Western blotting analysis of the CAIII protein level in the soleus muscle of the rats after electrical stimulation for 5 min, 10 min, and 20 min, respectively (in triplicate) (each group, N = 5). (C) Densitometric analysis of CAIII protein at 5 min, 10 min, and 20 min, respectively (each group, N = 5), after stimulation from the Western blot experiment. *P < 0.05, ***P < 0.001; β-actin is an internal reference control. CAIII = carbonic anhydrase III; CTRL = control group; SDS–PAGE = sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Downregulation of CAIII protein in the fatigued muscle

After the completion of the rat soleus muscle electrical stimulation fatigue test, we detected the levels of CAIII protein in muscles. The results revealed that the relative ratios of CAIII protein levels in 5-min, 10-min, and 20-min electrically stimulated fatigued muscles were 0.78 ± 0.26, 0.74 ± 0.30, and 0.59 ± 0.18, respectively, which had significant decrease compared with those of the control group ($P < 0.001$). In addition, there was a significant difference in CAIII protein levels between the 5-min and 20-min group ($P < 0.05$) (Fig. 4A and B).

Changes in MDA, lactate, and ATP levels during skeletal muscle fatigue

Biochemical analysis after the rat soleus muscle electrical stimulation fatigue test revealed that the content of MDA and lactic acid in the fatigued muscles significantly increased with prolonged stimulation time. Lactic acid levels for the 10-min and 20-min group were significantly increased compared with those of the control group ($P < 0.001$), and there was also a significant difference between the 5-min and 20-min group ($P < 0.05$) (Fig. 5A). Similarly, MDA levels for the 5-min, 10-min, and 20-min group were significantly increased compared with those for the control group ($P < 0.01$). Furthermore, there were also significant differences between the 5-min group and 20-min group ($P < 0.01$) and 10-min group and 20-min group ($P < 0.05$) (Fig. 5B). The ATP levels were significantly decreased in the 5-min, 10-min, and 20-min group compared with those in the control group ($P < 0.001$), whereas no significant differences were observed among the 5-min, 10-min, and 20-min group (Fig. 5).

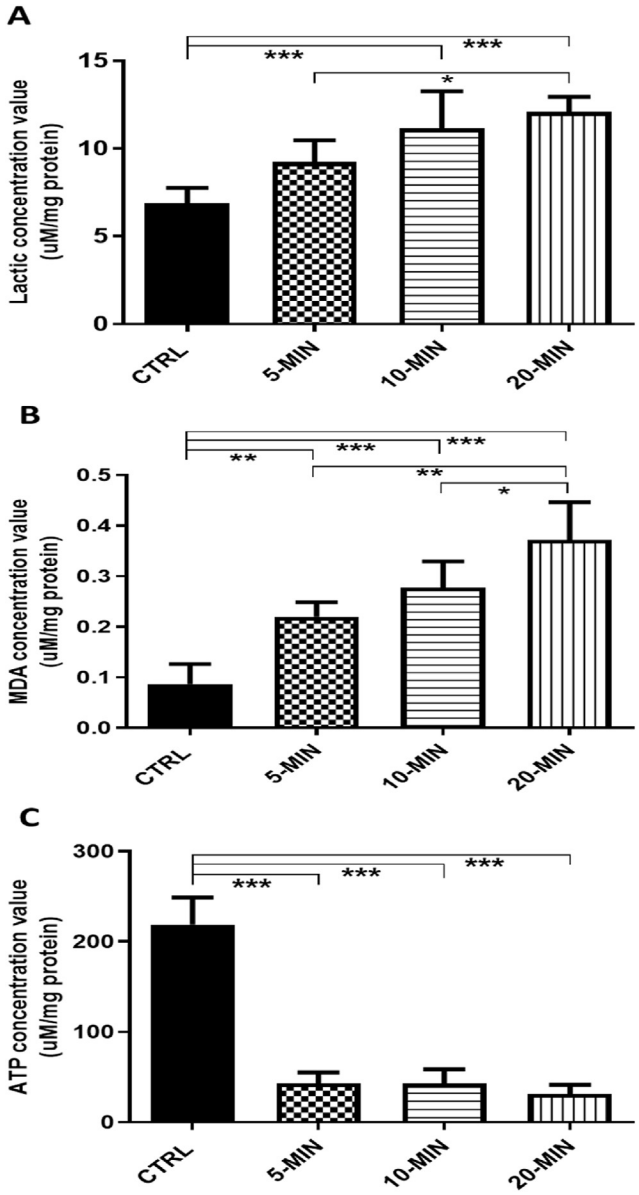

Figure 5. Biochemical indicators of muscle fatigue during electrical stimulation: (A) Lactic acid concentration, (B) MDA concentration, and (C) ATP levels in the soleus muscle after electrical stimulation for 5 min, 10 min, and 20 min, respectively (each group, N = 5). *P < 0.05, **P < 0.01, ***P < 0.001. MDA = malondialdehyde.

Effects of interference and overexpression of lentivirus transfection on muscle CAIII protein and mRNA levels

The soleus muscle was transfected by the empty lentivirus vector in situ and harvested at Days 7, 14, and 21, and an optimal fluorescence signal after transfection was observed at Day 21 (scale bar = 100 mm, Fig. 2). Interference and overexpression lentivirus vectors were injected into the rat soleus muscles to analyze the expression of CAIII protein and mRNA after 21 days. The results showed that the levels of CAIII protein (0.63 ± 0.38, $P < 0.001$) and mRNA (0.71 ± 0.13, $P < 0.001$) were significantly reduced in the RNAi group compared with those in the saline-injected group (NS) and empty lentivirus group (C-Virus). Moreover, the level of CAIII protein (1.28 ± 0.27, $P < 0.001$) and mRNA (1.31 ± 0.32, $P < 0.001$) in the CAIII-overexpressed lentivirus (GV358-CAIII)–injected group was significantly increased compared with that in the NS and C-Virus group (Fig. 6), suggesting that the lentivirus vectors for the suppression and overexpression of CAIII were successfully transfected and had stable expression.

Effect of CAIII suppression and overexpression on electrically stimulated muscle fatigue and muscle fatigue biochemical markers

The CAIII-downregulated and CAIII-overexpressed rat soleus muscles underwent electric stimulation muscle fatigue for 5, 10, and 20 min. In all the groups, soleus muscle force decreased with prolongation of stimulation time. However, the most significant decrease in muscle force was seen at 20 min in the RNAi group compared with that in the NS group (0.13 ± 0.00 vs 0.20 ± 0.01, respectively, $P < 0.05$, Fig. 7). The GV358-CAIII group showed the smallest decrease in the muscle force compared with the NS group (0.39 ± 0.02 vs 0.20 ± 0.01, respectively, $P < 0.05$, Fig. 7), indicating that CAIII gene overexpression could have an antifatigue effect. Furthermore, biochemical analysis of muscles revealed that the contents of MDA and lactic acid in fatigued muscles of the RNAi group were higher than those in the NS and C-Virus groups, whereas the ATP levels were lower than those in the NS and C-Virus groups ($P < 0.05$, Fig. 8). Meanwhile, we also found that the contents of MDA and lactic acid in the fatigued muscle were lower in the GV358-CAIII group than that those in the NS and C-Virus groups, but the ATP level was higher than that in the NS and C-Virus groups ($P < 0.05$, Fig. 8).

Discussion

CAs are a family of zinc-containing metalloproteases, and at least 16 CA isoforms have been described so far in mammals [8]. CAIII is one specific member of this family, which is remarkably abundant in type I skeletal muscle fibres; the slow contraction fibres (soleus muscle) were reported to have highly expression of CAIII proteins (about 2% of the net weight of cells), whereas type II skeletal muscle fibres, which are fast contractile fibres (tibialis anterior/extensor digitorum longus and pectoral muscle), were found to have lower expression of CAIII proteins [9]. Although CAIII is highly expressed in the skeletal muscle, its specific role in maintaining the physiological function of muscles is still under scrutiny.

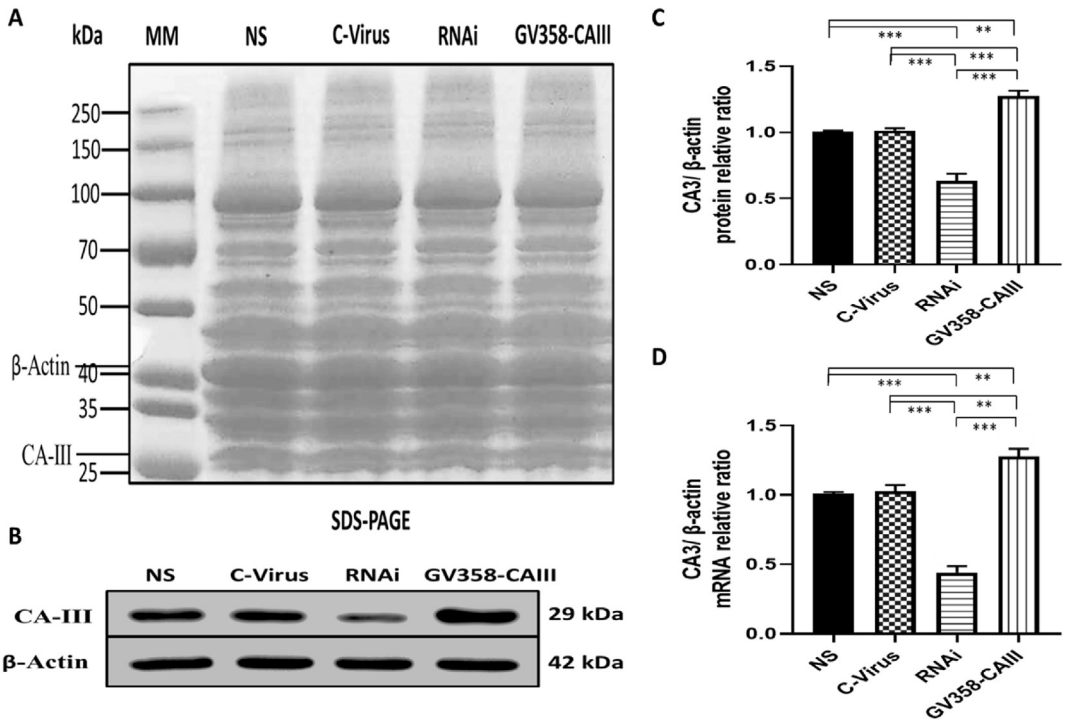

Figure 6. Changes of CAIII protein and mRNA expression in the skeletal muscle after lentivirus vector transfection: (A) SDS-PAGE gel to normalize the amount of protein for sample loading. (B) Western blotting analysis of the CAIII protein level. (C) Densitometric analysis of CAIII protein from Western blot results. (D) Quantitative real-time PCR analysis of CAIII mRNA in muscles after lentivirus vector transfection. ; **P < 0.01, ***P < 0.001; β-actin is an internal reference control. CAIII = carbonic anhydrase III; C-Virus = empty virus injection group; GV358-CAIII = overexpression virus injection; NS = physiological saline injection group; PCR = polymerase chain reaction; RNAi = interference virus injection group; SDS–PAGE = sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

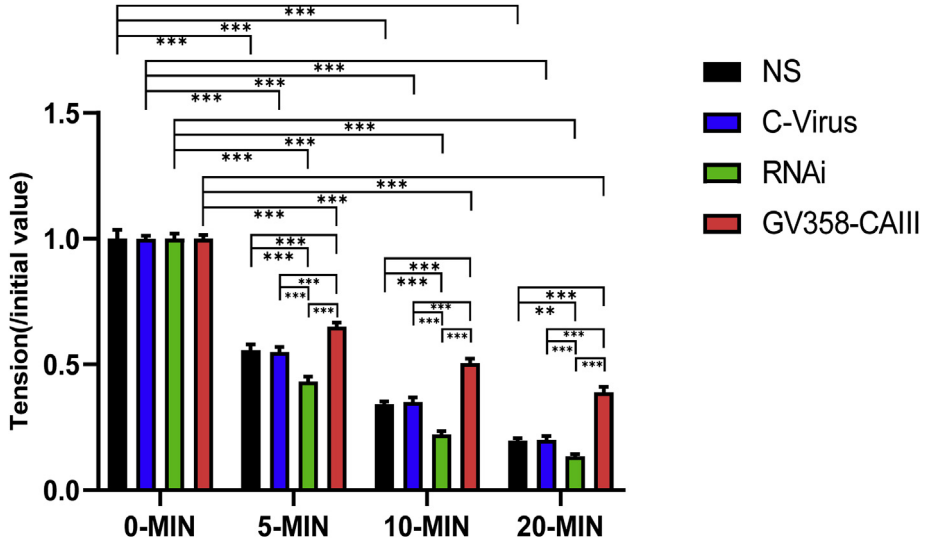

Figure 7. Changes in muscle force of the lentivirus vector–transfected soleus muscle after fatigue protocol. Gradual decrease in muscle force observed in all groups with increase in stimulation duration (each group, n = 5). ***P < 0.001. CAIII = carbonic anhydrase III; C-Virus = empty virus injection group; GV358-CAIII = overexpression virus injection; NS = physiological saline injection group; RNAi = interference virus injection group.

In this study, a rat model of induced fatigue of type I skeletal muscle fibers, such as the soleus muscle, was established using a low-frequency electrical stimulation. We observed that with the increase in the duration of electric stimulation resulted in reduced muscle tone and down-regulation of CAIII protein in the soleus muscle. This finding is consistent with previous reports that decrease in expression of CAIII in the skeletal muscle during vigorous exercise may cause muscle fatigue [9,11,23,24]. Similarly, we observed that during prolonged stimulation, there was an increase in accumulation of MDA and lactic acid, and the ATP levels fell rapidly to about 20% after 5-min stimulation and remained in this level until the end of the stimulation (Fig. 5). Linderger et al. [4] reported that under strenuous exercise, muscle fatigue is induced by accumulation of specific metabolites that could also change the intracellular pH. Feng and Jin [25] demonstrated that the functions of CAIII as the intracellular pH regulator are dependent on the range of intracellular pH, thus implying that the accumulation of lactic acid could decrease intracellular pH, and downregulation of CAIII hampered its ability of regulating intracellular pH; the synergy between these two processes might be involved in the

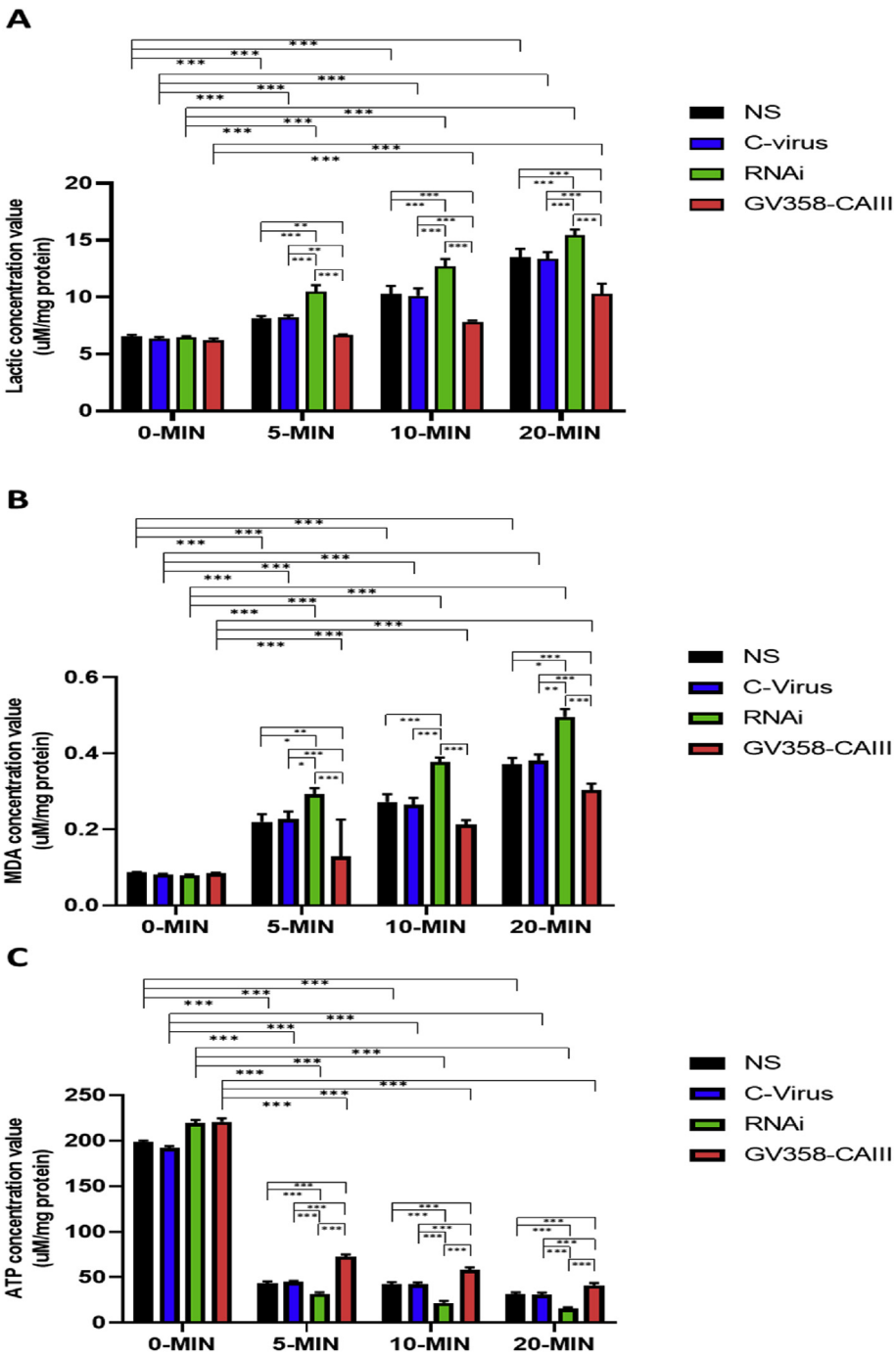

Figure 8. Changes of biochemical marker levels in the lentivirus vector–transfected soleus muscle after fatigue protocol. (A) Lactic acid concentration, (B) MDA concentration, and (C) ATP levels in the lentivirus vector–transfected soleus muscle after electric stimulation for duration of 5 min, 10 min, and 20 min, respectively (each group, n = 5). *P < 0.05, **P < 0.01, ***P < 0.001. CAIII = carbonic anhydrase III; C-Virus = empty virus injection group; GV358-CAIII = overexpression virus injection; MDA = malondialdehyde; NS = physiological saline injection group; RNAi = interference virus injection group.

occurrence of muscle fatigue.

Furthermore, to explore the underlying role of CAIII in muscle fatigue, we successfully constructed a CAIII-interfering lentivirus vector (RNAi) and CAIII-overexpressed lentivirus vector (GV358-CAIII) and injected them into the rat soleus muscles to suppress and overexpress CAIII in the muscles, respectively. The electrical stimulation results showed that in all the groups, muscle force gradually decreased with the prolongation of stimulation time. Among them, the least resistant to muscle fatigue was particularly noticed in the lentivirus RNAi group, whereas the lentivirus GV358-CAIII group demonstrated significantly high tolerance to muscle fatigue. Besides, the overexpressed CAIII group has lower MDA and lactic acid and higher ATP levels in the muscles. However, the RNAi group muscle had a higher accumulation of MDA and lactic acid and low ATP levels. As we know, the decrease of intracellular ATP levels in the skeletal muscle during exercise is another cause of muscle fatigue [6]. In addition, Liu et al. [11] performed muscle stimulation on CAIII-KO mouse muscles to reveal a decrease in ATP, Pi, and pH during stimulation and suggested that the absence of CAIII in skeletal muscles could impair mitochondrial ATP synthesis. Similarly, in this study, we observed that ATP and CAIII levels had been significantly reduced in short time (5 min, Figs. 4 and Fig 5-C). Moreover, after CAIII was overexpressed in muscles, the ATP level was higher than that in the NS and C-Virus groups but still very low (Fig. 8-C). It indicates that both CAIII and ATP might play an important role in the process of muscle fatigue. However, the exact relationships between CAIII and ATP, as well as which one plays a more prominent role in muscle fatigue, need to be further studied.

Another possible mechanism may be that during muscle fatigue, there

is an increase of ROS and involvement of CAIII in scavenging of free oxygen radicals during oxidative stress [5,26], although the exact mechanism of fatigue is still unclear. However, the effects of CAIII on ROS during muscle fatigue are beyond the scope of this study, and further research is required to elucidate the relationship between them. Although this study has demonstrated the active role of CAIII in muscle fatigue, it had some limitations such as lack of CAIII inhibitors such as methazolamide and zonisamide [27], lack of a rescue group (by injecting CAIII to evaluate the effect), and lack of female mice. However, RNA interference and gene recombination technology have been well established as a gene knockdown technique and are economical, fast, and efficient [28]. In addition, the effect of shorter duration of electrical stimulation on muscle force, ATP, and CAIII levels was not investigated in this study. However, Liu et al. [11] showed that when the gastrocnemius muscle from CAIII-KO mice underwent intense stimulation for a short duration of 2 min, it resulted in a decrease in ATP and PCr and fall in pH. Despite this, we are still able to conclude the effects of CAIII gene knockdown or overexpression on muscle fatigue and its possible mechanisms in this study.

The present study found that the occurrence of skeletal muscle fatigue may be related to the downregulation of muscle CAIII protein expression. The results of the study not only enrich the mechanism of muscle fatigue but also contribute to the in-depth study of the physiological function of CAIII in the skeletal muscle. Besides, this study might also provide a specific experimental basis for the exploration of new ways to eliminate skeletal muscle fatigue.

Conclusion

In this study, a rat model of skeletal muscle fatigue was established by electrical stimulation. It was found that electrical stimulation of soleus muscle fatigue could downregulate CAIII protein expression in muscles and decrease ATP levels. In addition, it resulted in the accumulation of lactic acid and MDA. Moreover, genetic studies, such as suppression and overexpression, of the CAIII gene demonstrated that muscle force and muscle fatigue are dependent on CAIII expression in the soleus muscles. However, the mechanism underlying CAIII protein utilization and its genetic suppression during muscle fatigue are still unclear and warrant further investigation.

Author contributions

A.B.S., H.F., and X.S. contributed to conception of and designed the study. H.F., M.L., and A.B.S. performed animal studies. H.F. performed data acquisition and tissue analysis. A.B.S., H.F., M.L., and X.S. performed data analysis. S.C. and P.S. supervised the study. X.S. acquired the research funding; A.B.S. and M.L. drafted the manuscript. S.C., P.S., and X.S. revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Conflicts of Interest

The authors have no conflicts of interest to disclose in relation to this article.

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