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Muscle plasticity related to changes in tubulin and αB-crystallin levels induced by eccentric contraction in rat skeletal muscles

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We used the model of eccentric contraction of the hindlimb muscle by Ochi et al. to examine the role of eccentric contraction in muscle plasticity. This model aims to focus on stimulated skeletal muscle responses by measuring tissue weights and tracing the quantities of αB-crystallin and tubulin. The medial gastrocnemius muscle (GCM) responded to electrically induced eccentric contraction (EIEC) with significant increases in tissue weight ($p < 0.01$) and the ratio of tissue weight to body weight ($p < 0.05$); however, there was a decrease in soleus muscle weight after EIEC. EIEC in the GCM caused contractile-induced sustenance of the traced proteins, but the soleus muscle exhibited a remarkable decrease in α-tubulin and a 19% decrease in αB-crystallin. EIEC caused fast-to-slow myosin heavy chain (MHC) isoform type-oriented shift within both the GCM and soleus muscle. These results have shown that different MHC isoform type-expressing slow and fast muscles commonly undergo fast-to-slow type MHC isoform transformation. This suggests that different levels of EIEC affected each of the slow and fast muscles to induce different quantitative changes in the expression of αB-crystallin and α-tubulin.

Keywords: heat shock protein, cytoskeleton, skeletal muscle, myosin heavy chain, isoform type transition, electrically induced eccentric contraction

Introduction

A characteristic feature of skeletal muscles is their adaptability (19). Metabolic differences among different muscle types are adapted to meet their functional demands or environmental stimuli, including mechanical forces, endocrine hormones, nutrition, muscle damage, aging, exercise, and other factors (16, 17, 20, 33). This remarkable capacity of skeletal muscle is usually associated with significant changes in muscle mass. Skeletal muscle shows its phenotypic adaptation as atrophy or hypertrophy (8). Low-frequency stimulated muscles increase the combination of slower myosin heavy chain (MHC) isoforms (7). Unloaded soleus muscle fibers show slow-to-fast MHC transition in a time-dependent manner during atrophy (34). Muscle hypertrophy can be artificially induced in animal models using eccentric contraction (22, 23).

Eccentric contraction happens when the lengthening force statue of muscles exceeds its normal contraction ability (18, 25). Eccentric contraction induces stretching or a continuously

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stretched state in muscles. Eccentric contraction can also have negative or positive effects on skeletal muscles (8, 12, 36). It is well known that stretching can not only cause muscle damage but also prevent muscle atrophy depending on the extent of intensity (5). Baewer et al. (5) emphasized that stretching is indispensably important for maintaining or ameliorating the structural solidity of muscle fibers. However, there are relatively few studies on the positive effects of electrically induced eccentric contraction (EIEC) in hindlimb muscles.

αB-crystallin, one of heat shock proteins (HSPs), is closely associated with the cytoskeleton and tubulin to perform its function by forming a system that induces muscle adaptability (2–4, 30). Microtubules, which consist of tubulin, are associated with different fibril types depending on muscle fibers and their protein contents, and their plasticity is controlled by electrical activity (29). We previously traced these proteins to determine whether the system formed by αB-crystallin and its substrate (tubulin) responds to mechanical environmental demands resulting in morphological and biochemical responses, which lead to adaptive transformation of the skeletal muscle (13, 15).

In this study, we focused on tracing the quantitative relationship between MHC isoforms and the system-forming proteins to determine if EIEC affects fast and slow muscles in relation to their reactive modalities. We subsequently examined the effect of EIEC on positive muscle adaptation, which contributes to muscle hypertrophy. This study provides information that may help improve our understanding of the mechanism of adaptability of skeletal muscle to EIEC as an important means to promote positive muscle adaptability.

Materials and Methods

Animal care

Eleven-week-old adult male Wistar rats weighing 280–350 g were used for this study. Rats were randomly assigned to the EIEC group ($n = 6$) and the Control group ($n = 6$) after 1 week for their adaptations. All animals were given standard rat chow and water *ad libitum*. They were housed at 22–24 °C with a 12:12-h light–dark cycle. Animal use and maintenance were approved by The University of Tokyo Animal Care and Use Committee, Tokyo, Japan.

EIEC model and sample acquisition

The EIEC model was previously described in detail by Ochi et al. (26) (Fig. 1). Under anesthesia, rats in the EIEC group were firmly fixed on a platform in the prone position. Plantarflex of the triceps surae muscle was induced by electrical stimulation, with the stimulus voltage set at submaximal isometric twitch torque (pulse duration, 0.4 ms; frequency, 100 Hz; and voltage, 35 V). The speed and the range of forced lengthening were 30°/s and from 0° to 45°, respectively. In the middle of each 3 s of submaximal isokinetic twitch torque, which produced the plantarflex of the triceps surae muscle, forced isokinetic dorsiflexion was induced from a footplate to the ankle joint for eccentric contraction of the triceps surae muscle (Fig. 2). After eccentric contraction every 2 days for 20 days in total (10 sessions of stimulation) (Fig. 2), the EIEC rats were euthanized by cervical dislocation under anesthesia induced by isoflurane (50 ml/kg body mass). Whole gastrocnemius muscle (GCM) and soleus muscle were isolated from EIEC and Control rats, and those muscles were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

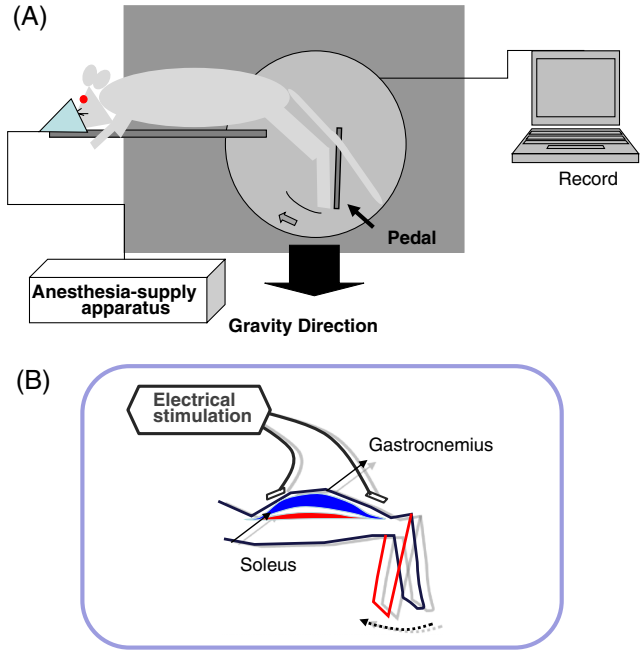

Fig. 1. Device used for electrical stimulation of the plantarflex muscles for eccentric contraction over a period of 21 days in rats. (A) Depiction of the prone position of the rat for eccentric contraction. Electrical stimulation induces plantarflex of the ankle, while the pedal induces dorsiflexion of the ankle. (B) Relationship between the plantarflex muscles (medial gastrocnemius and soleus muscles) and forcible eccentric contraction (dorsiflexion)

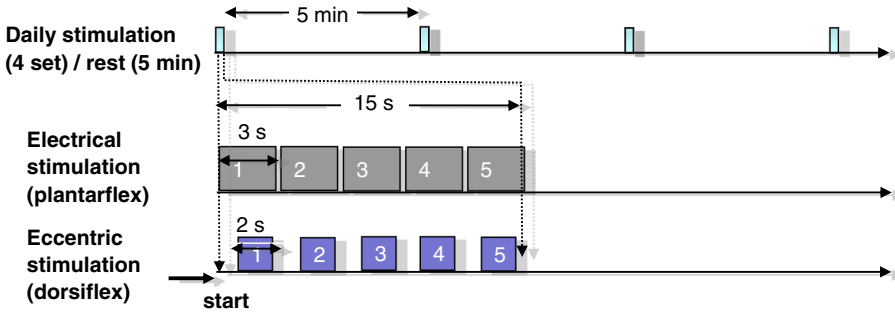

Fig. 2. Exercise program created by electrically induced eccentric contraction (EIEC). EIEC of the soleus muscle and gastrocnemius muscle (GCM) induced dorsiflexion of the ankle after the first 1 s of every 3 s electrical stimulation. The plantarflex for forcibly dorsiflexed EIEC was induced five times per session and was repeated four times/day

Preparation of muscle samples

The isolated whole muscles were crushed in liquid nitrogen and solubilized in a low salt buffer containing 20 mM potassium chloride (KCI), 2 mM sodium phosphate (pH 6.8), 2 mM ethylene glycol tetraacetic acid, 5 mM ethylene diamine tetraacetic acid, 20 mM sodium fluoride, and 1 mM sodium orthovanadate; protease inhibitors containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor; and phosphatase inhibitors containing 100 nM okadaic acid and 10 mM sodium β-glycerophosphate. The homogenate was solubilized with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer [2 mM sodium phosphate (pH 6.8), 2% SDS, and 16% 2-mercaptoethanol] and boiled for 2 min. The homogenate was centrifuged at 15,000 × *g*

at 4 °C for 10 min. The supernatant was collected, and the protein concentration was determined using a protein determination kit (Bio-Rad, Richmond, CA, USA).

SDS polyacrylamide gel electrophoresis (PAGE) and western blotting

Polyacrylamide gels (8.5–12%) were used as running gels to detect αB-crystallin, tubulin, and MHC proteins. As stacking gels, we commonly used 3.5% (w/v) polyacrylamide gels. To separate MHC isoforms, 8.5% polyacrylamide gels were loaded with 1.6 μg of sample of protein homogenates in 30% glycerol solution, described in the *Preparation of muscle samples* section, which were electrophoresed over a 24-h period at a constant voltage of 150 V and 4 °C (1). Gels were stained with Coomassie Brilliant Blue R-250 dye (Sigma-Aldrich, St. Louis, MO, USA). The relative percentages of MHC isoform contents were calculated using ImageJ (National Institutes of Health, Rockville Pike Bethesda, MD, USA). The markers used were a mixture of extracted soleus muscle and GCM, because not all MHC isoforms in the mixture overlap with the MHC isoforms in the soleus muscle and GCM.

For αB-crystallin and α-tubulin analysis, 12% polyacrylamide gels were used as running gels. Gels were loaded with 0.5–7 μg and 9–38 μg of the extracted muscle samples to measure the absolute quantity of αB-crystallin and α-tubulin, respectively, in the muscle samples.

The following primary antibodies were used: anti-α-tubulin (1:1,000, Sigma-Aldrich, St. Louis, MO, USA); anti-αB-crystallin (1:5,000, raised in rabbit against the C-terminal 10 peptides [(SH) KPAVTAAPKK] of human αB-crystallin); and anti-heat shock cognate (HSC) 70 (1:1,000, Santa Cruz Bioscience, CA, USA). After incubation with secondary antibodies, the membrane was incubated with an enhanced chemiluminescence kit (Amersham Biosciences Co., Buckinghamshire, UK). αB-crystallin was expressed in *Escherichia coli* (35) and purified by protein chromatography, and tubulin (phosphocellulose column purified tubulin) was purified from the porcine brain (13). The purified proteins (αB-crystallin, 5, 10, 20, 30, and 40 ng; α-tubulin, 1, 3, 5, 7, 9, and 11 ng) were used as standards for each protein because the relationship between the gradual contents of each purified protein and the linearity of band intensity is proportional. In addition, the different quantities of extracted proteins from 0.5 to 38 μg of the muscles, with each corresponding protein standard, were loaded on the same gel to estimate the exact quantity of each protein in the extracted muscle samples. The content of αB-crystallin estimated from the 0.5–7 μg of extracted muscle samples and α-tubulin estimated from the 9–38 μg of extracted muscle samples were expressed as ng/μg.

Examination of the loading quantity

Gels containing 7 μg of extracted muscle samples ($n = 12$ for each muscle) were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO, USA) to determine if the same quantity of each extracted muscle sample was loaded. Furthermore, 20 μg/lane of HSC 70 was used as a control, as described in the immunoblotting methods in the *SDS polyacrylamide gel electrophoresis (PAGE) and western blotting* section (Fig. 3C) (30).

Statistical analysis

All data were presented as means ± standard deviation (SD), and differences between the Control and EIEC groups were analyzed by the independent *t*-test (SPSS Ver 18.0, Chicago, IL, USA). The level of statistical significance was set at as $p < 0.05$ for all analyses.

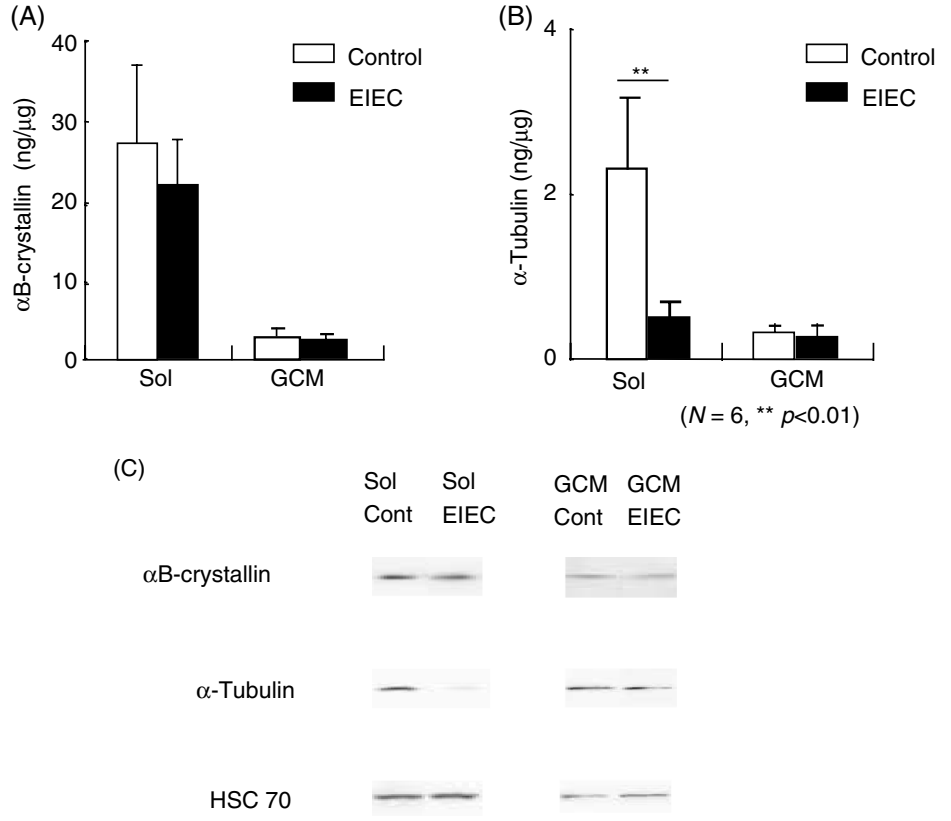

Fig. 3. Response of αB-crystallin and α-tubulin to electrically induced eccentric contraction (EIEC) over the period of 21 days. The rat was anesthetized using isoflurane during EIEC. (A) αB-crystallin quantity in the soleus muscle and medial GCM. (B) α-Tubulin quantity in the soleus muscle and GCM. α-Tubulin quantity in the soleus muscle was dramatically reduced by EIEC (**$p < 0.01$). α-Tubulin in GCM EIEC did not significantly change. (C) Quantified western blot bands of αB-crystallin, α-tubulin, and heat shock cognate (HSC) 70 as control were shown. $N = 6$. Sol, soleus muscle; GCM, medial gastrocnemius muscle; Sol Cont, soleus muscle control; Sol EIEC, soleus muscle in EIEC; GCM Cont, medial gastrocnemius muscle control; and GCM EIEC, medial gastrocnemius muscle in EIEC

Results

Body and muscle weight

The body weight (BW) and tissue weight (TW) of the Control and EIEC groups were measured (Table I), and the result revealed that EIEC did not change the BW of the animals. The TW of the GCM in the EIEC group was significantly increased compared with the Control group ($p < 0.01$). The ratio between the tissue and body weights (TW/BW) in the GCM was also significantly hypertrophied compared with the Control group ($p < 0.05$) (Table I); however, there were decreases in TW (6%) and TW/BW (5%) in the soleus muscle.

Changes in MHC isoforms

In the GCM, MHC IIa was increased ($p < 0.05$) and MHC IIb was decreased ($p < 0.01$) after EIEC (Table II). About 20% increase in MHC I was observed, although no significant change occurred after EIEC in the GCM (Table II). Following EIEC in the soleus muscle, there was a

Table I. Body and muscle tissue weights of eccentric contraction stimulation

			Control	Experiment
E.C.S.	BW (g)		337.6 ± 7.2	336.5 ± 9.9
	TW (mg)	Medial gastrocnemius	753.5 ± 16.7	820.3 ± 48.4**
	TW/BW (mg/g)		2.23 ± 0.05	2.44 ± 0.16*
	TW (mg)	Soleus	135.4 ± 5.2	127.3 ± 23.4
	TW/BW (mg/g)		0.40 ± 0.02	0.38 ± 0.07

$N = 6$. Values are means ± SD. E.C.S. = eccentric contraction stimulation, BW = body weight, TW = tissue weight, Medial gastrocnemius = medial gastrocnemius muscle, Soleus = soleus muscle. *$p < 0.05$, **$p < 0.01$: statistical significances to its Control

Table II. Myosin heavy chain isoforms

			%			
			I	IIa	IId/x	IIb
E.C.S.	Medial gastrocnemius	Control	21.8 ± 6.1	45.5 ± 4.5	0 ± 0	32.7 ± 6.0
		Experiment	26.1 ± 3.1	53.2 ± 4.8*	0 ± 0	15.6 ± 2.4**
	Soleus	Control	84.7 ± 4.0	15.3 ± 4.0	0 ± 0	0 ± 0
		Experiment	95.0 ± 4.0**	5.0 ± 4.0**	0 ± 0	0 ± 0

$N = 6$. Values are means ± SD. E.C.S. = eccentric contraction stimulation. *$p < 0.05$, **$p < 0.01$: statistical significances to its Control

significant increase ($p < 0.01$) in MHC I and a decrease ($p < 0.01$) in MHC IIa, with no changes in MHC IId and MHC IIb. Therefore, EIEC in both the soleus muscle and the GCM showed fast-to-slow MHC isoform transformation within MHC isoforms in this study.

αB-crystallin content in EIEC muscles

Absolute quantities of αB-crystallin in the GCM and soleus muscle were measured by western blotting (Fig. 3A), with no statistically significant differences between the EIEC and Control groups. The soleus muscle has about eight times more αB-crystallin compared with the GCM. The quantitative difference of αB-crystallin between the soleus muscle and the GCM changed about 7.9-fold following EIEC. Figure 3C indicates immunoblotting bands of αB-crystallin in the GCM and soleus muscle between the Control and EIEC groups.

α-Tubulin content in EIEC muscles

The immunoblotting bands for the absolute quantity of α-tubulin in the GCM and soleus muscle following EIEC are shown in Fig. 3C. α-Tubulin content in the GCM in the EIEC group showed no significant change compared with the Control group, even though it tended

to reduce to approximately 33% of the Control group following 10 sessions of EIEC. Interestingly, there was an almost 80% decrease in the absolute quantity of α-tubulin in the soleus muscle following EIEC ($p < 0.01$) (Fig. 3B). The absolute quantity of α-tubulin in the soleus muscle was almost 7.7-fold more than that in the GCM. This difference was diminished around 2.5-fold owing to the remarkable decrease in the α-tubulin quantity of the soleus muscle following EIEC.

Discussion

The novel findings from this study were that EIEC induced a significant hypertrophy ($p < 0.01$) with remarkable fast-to-slow MHC isoform-orientated shift in the fast-twitch GCM; however, a slow MHC isoform-orientated shift without hypertrophy occurred in the soleus muscle. Thus, we provide previously unknown information that EIEC in the GCM caused contractile-induced sustenance of the traced proteins; however, the soleus muscle had a remarkable decrease ($p < 0.01$) in α-tubulin levels with approximately 19% decrease in αB-crystallin content. Different levels of EIEC causing passive and active stretching possibly affected both slow and fast muscles, respectively, which would likely be reflected in different quantitative patterns of αB-crystallin and α-tubulin in each muscle.

In this study, we used GCM (MHC I, MHC IIa, and MHC IIb) and whole soleus muscle (MHC I and MHC IIa) (15). The GCM and soleus muscle have relatively high proportions of slow-type shifted MHC isoforms, whose changes were efficiently detected in this study (Table II). Change in the content of MHC isoforms does not necessarily correlate with the number of specific fibers or the proportion of different fiber types. Thus, we used MHC isoforms as a standard *per se* (28, 31), not as a ratio of slow or fast twitch fibers in the muscles that show the physiological, metabolic, and functional properties of skeletal muscles (6). For MHC isoforms in slow and fast muscles, their isoform transitions show the following order MHC I↔MHC IIa↔MHC IId/x↔MHC IIb (9). Ralston et al. (29) reported that 20 Hz stimulation induced a slow type I pattern in extensor digitorum longus fibers (fast) muscle, but stimulation with 150 Hz only partially succeeded in inducing a type II pattern in the soleus (slow) muscle. The difference in frequency of electrical stimulation (100 Hz of EIEC used in this study and 150 Hz of EIEC used in Ralston et al.'s study) might be responsible for the different results in this study compared with those of Ralston et al. (29).

In this EIEC model, actively stretched contraction of GCM by electrical stimulation caused muscle hypertrophy (Table I). Our results suggest that not only the stretch but also active muscle contraction is indispensable for muscle hypertrophy, because no hypertrophy occurred in the soleus muscle, although the different constitutional MHC isoforms between the soleus muscle and GCM possibly affected the results in this study.

In the EIEC model, forcible electrical stimulation indiscriminatingly permeated all intracellular organelles, driving a massive efflux of Ca^{2+} into the cytosol, which contributes to muscular activities (14). Especially, it is well known that eccentric contraction generates the largest tension among muscle contraction types (18). We suggest that the largest mechanical stress seen in this study occurred by eccentric contraction and not by other forms of contraction, and that it efficiently responded to muscular environmental changes, including muscle hypertrophy. It is possible that different responses within the sarcomere produced different results between the soleus muscle and GCM in this study, because the active mechanical force from eccentric contraction was directed to the Z-line of the

sarcomere, which is a starting point for various signal cascades associated with muscle plasticity (10).

Our findings in this study are consistent with a previous report that the overall content of αB-crystallin does not change, but it is relatively highly phosphorylated and its binding rate with the cytoskeleton is increased by stimulation following eccentric contraction (11). Frankenberg et al. (11) have also suggested that these properties of αB-crystallin are probably dependent on the time of tissue sampling. Ochi et al. (26) reported that β-titin, one of the strong potential candidates for change induced by eccentric contraction, was not affected by eccentric contraction. Studies showed that HSP27, αB-crystallin, HSP70, and HSP72 have different expression patterns following eccentric contraction (21, 24, 27, 32). These results suggest that eccentric contraction probably induces individually different responses in each chaperone protein.

In this study, αB-crystallin did not respond in a predictable manner to EIEC, and α-tubulin unexpectedly showed a remarkable decrease in the soleus muscle; however, no significant changes of these proteins occurred in the GCM. These results may be caused by a number of mechanisms. Different degrees of electrical stimulation to the skeletal muscles could cause variations in the quantity of tubulin per microtubule (29). The active and direct electrical stimulation of the GCM sustains tubulin levels. As an unexpected novel finding of this study, this difference suggests that different levels of electrical stimulation might be an important factor for controlling the expression of tubulin and that α-tubulin is potentially controlled by neuronal stimulation. Ralston et al. (29) reported that different frequencies of electrical stimulation induced different densities and orientations of microtubules in the soleus (which dominantly has MHC I) and extensor digitorum longus (which dominantly has MHC II) muscles, whereby 150 Hz (100 Hz in this study) stimulation applied to the fast muscle preserved the pattern/quantity of microtubules; however, microtubules in the slow muscle under the same stimulation showed thinner and were fewer in quantity. We have hypothesized that a decrease in the pool of tubulin dimers rather than a decrease in microtubules is caused by higher frequencies of electrical stimulation comparing with the lower ones.

Limitation

Further studies, such as biomechanics at the single muscle fiber level, are needed to determine the functional quality of skeletal muscle and to understand the efficiency of eccentric contraction-mediated muscular plasticity.

Conclusion

In this study, we found that EIEC induced the muscle hypertrophy and sustained the expression of αB-crystallin and tubulin in the GCM. EIEC induced fast-to-slow MHC isoform-oriented shift in fast muscle; however, only slow MHC isoform-oriented shift occurred without significant effects on muscle weight in the soleus muscle. There was a remarkable (19%) decrease ($p < 0.01$) in α-tubulin levels in the simultaneously stimulated soleus muscle. These results presumably indicate less bearing load in eccentric contraction comparing with simply contracted and stretched stimulation. These findings may help to explain the phenomenon that (i) slow and fast MHC isoforms have different responses to

EIEC for those specific muscle adaptabilities and (ii) suitable mechanical force by EIEC associated with electrical stimulation controlled the tubulin expression and sustained the expression of αB-crystallin and tubulin, which contributed to positive muscular adaptability.

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Conflict of interest

The authors have no conflict of interest.

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