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Basic nutritional investigation

Ingestion of soy protein isolate attenuates eccentric contraction-induced force depression and muscle proteolysis via inhibition of calpain-1 activation in rat fast-twitch skeletal muscle

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ABSTRACT

Objective: Eccentric contraction (ECC) is a contraction in which skeletal muscles are stretched while contracting. The aim of this study was to determine how ingestion of soy protein isolate (SPI) or animal-based proteins affect force deficit, calpain activation, and proteolysis of calcium ion (Ca^{2+})-regulatory proteins in rat fast-twitch muscles subjected to ECC.

Methods: In the first experiment, male Wistar rats were randomly assigned to a control and an SPI group, which were fed a 20% casein and a 20% SPI diet, respectively, for 28 d before the ECC protocol. Anterior crural muscles underwent 200 repeated ECCs and were excised 3 d later. In the second experiment, half of the SPI rats were given water containing N^G-nitro-L-arginine-methyl ester (L-NAME), an inhibitor of nitric oxide synthase, for 3 d of recovery after ECC.

Results: SPI ingestion attenuated ECC-induced force deficit, proteolysis of Ca^{2+}-regulatory proteins, and autolysis of calpain-1. Co-ingestion of L-NAME inhibited SPI-associated increases in nitrite and nitrate levels and negated the force recovery effects of SPI.

Conclusion: These results suggest that SPI ingestion inhibits ECC-elicited force deficit and proteolysis of Ca^{2+} regulatory proteins, which is caused by inhibited activation of calpain-1 via increased nitric oxide production.

Introduction

Eccentric contraction (ECC) is a contraction in which skeletal muscles are stretched while contracting. ECC tends to result in a larger and longer-lasting force deficit than concentric and isometric contractions [1,2]. After ECC, there often is muscle injury that is characterized by proteolysis of muscle proteins, damage to sarcomeres and plasma membranes, inflammation and swelling, and delayed-onset muscle soreness and stiffness [1,3–6]. Although it remains unclear to what extent these changes contribute to ECC-induced force deficit, recent studies suggest that large and long-lasting force deficit associated with ECC is, in large part, due to proteolysis of muscle proteins (e.g., calcium ion [Ca^{2+}]-regulatory proteins) critical for muscle contraction [2,5,7–9] and that the proteolysis primarily involves the Ca^{2+}-regulated cysteine proteases calpains [5,6,8,10]. Daily physical exercise, including training of competitive sports, includes an eccentric component to some extent. It appears likely, therefore, that inhibiting the negative effects of ECC is an effective strategy for overcoming reduced muscle performance that results from physical exercise.

Reactive oxygen and nitrogen species (ROS/RNS) are moderately generated in the resting state in cells. In skeletal muscles, the dominant ROS and RNS are superoxide and nitric oxide (NO), respectively. Some superoxide is generated through incomplete reduction of oxygen in the mitochondria [11]; whereas the Ca^{2+}-calmodulin complex activates NO synthase (NOS) [12]. Thus, ROS/RNS production is markedly augmented with muscle

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contraction, which results in the increase of both the oxygen demand and the Ca^{2+} concentration in the cells.

Modest and transient increases of ROS/RNS play an essential role in cellular signals involved in altered force production, elevated glucose uptake, and muscle adaptation to physical exercise [11,13,14]; whereas high and/or prolonged levels cause impaired function of Ca^{2+}-regulatory proteins and/or the contractile apparatus via chemical modifications [11,15]. Previous in vitro studies regarding the effect of NO on calpains have revealed that application of NO donors mitigates calpain activation [16,17], which is mediated through *S*-nitrosylation of the enzyme [16]. Based on these findings and the fact that L-arginine (ARG) is a precursor of NO, we examined the effect of ingestion of ARG on calpain in rat fast-twitch muscles undergoing ECC and found that ARG ingestion was capable of attenuating ECC-induced calpain activation and force deficit [18]. These effects of ARG ingestion also have been observed in rat slow-twitch muscles [19].

Soy protein is a widely available plant-based protein, and as with animal-based proteins (e.g., beef, egg, and milk), its ingestion has been shown to stimulate muscle protein synthesis both at rest and after repeated contractions [20]. In view of previous results on ARG ingestion [18], the amino acid composition of soy protein isolate (SPI) is of great interest. The fact that SPI contains more than twice the ARG compared with animal-based proteins [21] raises the hypothesis that, as does ARG ingestion, SPI ingestion could lessen the deleterious influence of ECC that is caused by calpain activation. However, to our knowledge, no studies have yet to explore this point.

The aim of this study was to determine how ingestion of SPI or animal-based proteins affects force deficit, calpain activation, and proteolysis of Ca^{2+}-regulatory proteins in rat fast-twitch muscles subjected to ECC. The experiments conducted with rat fast-twitch muscles provide evidence in favor of the aforementioned hypothesis and reveal that inhibition of calpain activation is ascribable to increases in NO production.

Materials and methods

Animal care

In this study, two separate experiments (1 and 2) were performed. In these experiments, 4-wk-old male Wistar rats (at the beginning of the experiment; Charles River Laboratories, Yokohama, Japan) were used. They were housed in a room with controlled environment (~24°C), with 12-h light/dark cycles, and were provided with water and chow (see below) ad libitum. An intraperitoneal injection of a mixture of medetomidine (0.4 mg/kg body weight [BW]), midazolam (2 mg/kg BW), and butorphanol (2.5 mg/kg BW) was used for anesthesia. At the end of the experiments, the rats were sacrificed with an overdose of pentobarbital sodium (200 mg/kg BW), followed by cervical dislocation. All procedures performed in this study were approved by the Animal Experimental Committee of Okayama Prefectural University.

Experiment 1

SPI ingestion and ECC protocol

The aim of experiment 1 was to investigate the effects of two forms of protein (SPI and casein) on force deficit, calpain activation, and proteolysis of Ca^{2+}-regulatory proteins in rat fast-twitch muscles subjected to ECC. The rats were randomly assigned to a control (CON) and an SPI group (n = 6 for each group) and were fed an AIN-93 G-based diet (Oriental Yeast, Osaka, Japan) containing 20% casein (for CON rats) or 20% SPI (for SPI rats) ad libitum for 28 d before the ECC protocol [22]. Under anaesthesia, ECCs were elicited in situ in the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles by stimulating the left peroneal nerve using a 1-s train of a 1-ms pulse at 50 Hz during forced plantar flexion (150-degree angular movement at 150 degree/s) [5]. ECCs were repeated every 4s for 200 cycles. Three days after the ECCs, EDL muscles of the experimental (left) and contralateral (right) legs were excised under anesthesia. CON and SPI rats were fed the respective diets during 3 d of recovery.

In vitro measurements of isometric force

Isolated EDL muscles were mounted in a chamber between a force transducer and an adjustable holder and were perfused with a standard Krebs-Ringer solution at 30°C [23,24]. Isometric contractions were elicited by direct stimulation at 20 and 80 Hz with a 1-s train of a 1-ms pulse. The forces were normalized to cross-

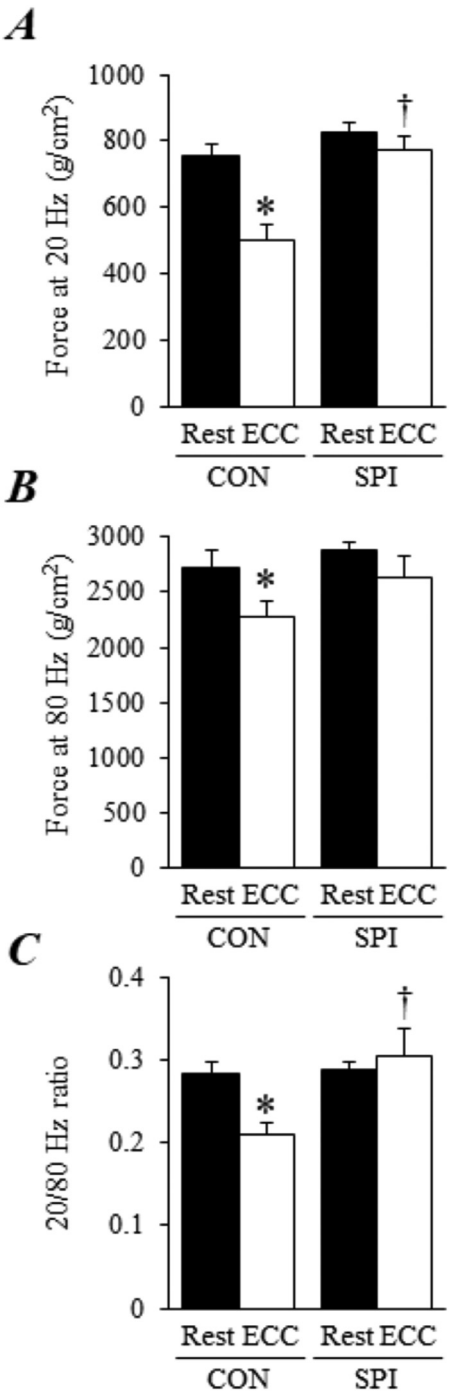

Fig. 1. Effect of SPI ingestion and ECC on specific isometric force at 20 Hz (A) and 80 Hz (B) and force ratio (C). CON and SPI rats were fed a 20% casein and a 20% SPI diet, respectively, for 28 d before the ECC protocol. ECCs were repeated in the anterior muscles of the left hindlimb for 200 cycles. Three days after ECC, tetanic contraction of isolated extensor digitorum longus muscles was evoked by electrical stimulation at 20 and 80 Hz, and the ratio of force at 20 Hz to that at 80 Hz (20/80 Hz ratio) was calculated. Values are means ± SEM (n = 6 for each muscle). CON, control; ECC, eccentric contraction, SPI, soy protein isolate. $^{*}P < 0.05$ versus rested muscles. $^{\dagger}P < 0.05$ versus ECC muscles from CON rats.

sectional area, which was computed as wet muscle weight divided by the product of muscle length and density (1.06 mg/mm^{-3}) [25].

Calpastatin activity

The tissue was homogenized in 9 vol (vol mass-) of an ice-cold buffer composed of 20 mM Tris/HCl (pH 7.4), 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol (DTT), 14 μM pepstatin A, and 10 μg/mL 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBF). Protein content of the resultant homogenate was determined using the Bradford assay [26]. The activity of calpastatin, an endogenous inhibitor of calpain, was fluorometrically measured [10]. Aliquots of homogenates were heated at 100°C for 10 min to inactivate endogenous calpains and other proteases. Muscle homogenates obtained from standard EDL muscles with or without the heated homogenates were preincubated at 37°C for 10 min in a buffer solution consisting of 20 mM Tris/HCl (pH 7.4), 1 mM DTT, 14 μM pepstatin A, 10 μg/mL AEBF, and 5 mM calcium chloride ($CaCl_2$). The reaction was started by adding *N*-Succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC), and the fluorescence of liberated AMC was monitored for 30 min. A control assay was performed in the presence of 10 mM EDTA and 10 mM EGTA, but without $CaCl_2$. Calpastatin activity was evaluated by the extent to which calpain activity in the standard samples was inhibited.

Immunoblotting

Equal amounts of proteins were electrophoretically separated and transferred to polyvinylidene difluoride membranes [18]. The membranes were incubated with the following primary antibodies: antiryanodine receptor (RyR) 1/2 (1:2,500 dilution; MA-925; Thermo Fisher Scientific, Waltham, MA, USA), antidihydropyridine receptor (DHPR) α1 subunit (1:1,000 dilution; MA3-920; Thermo Fisher Scientific), antijunctophilin (JP) 1 (1:1,000 dilution; 40-5100; Thermo Fisher Scientific), anticalpain-1 (1:1,000 dilution; C0355; Sigma, St. Louis, MO, USA), and anticalpastatin (1:200 dilution; sc-7561; Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were then incubated with the following secondary antibodies: Sc-2004 for JP1 (1:5,000 dilution; Santa Cruz Biotechnology), sc-2020 for calpastatin (1:5,000 dilution; Santa Cruz Biotechnology), and P0260 for DHPR, RyR, and calpain-1 (1:5,000 dilution; Agilent Technologies, Santa Clara, CA, USA). To quantify total proteins on the membrane, the membrane was stained with Coomassie Blue R. Immunoreactive and Coomassie-stained bands were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the levels of the four proteins investigated (RyR, DHPR, JP1, and calpastatin) were normalized to the total proteins.

Experiment 2

SPI ingestion, ECC protocol, and NOS inhibitor administration

Experiment 1 revealed that SPI ingestion was able to lessen the ECC-elicited force deficit and proteolysis of Ca^{2+} regulatory proteins via inhibition of calpain-1 activation (see Results section). Experiment 2 was performed to elucidate whether endogenously produced NO contributes to the observed changes. The rats were randomly assigned to a CON (n = 5) or an SPI (n = 10) group. The procedures for SPI ingestion and ECC were similar to those used in experiment 1. Half of the SPI rats (n = 5) were given water containing the non-selective NOS inhibitor L-NAME (Cayman Chemical, Ann Arbor, MI, USA) ad libitum for 3 d of recovery after ECC (average ingestion of 75 mg/kg BW/d^{-1}).

In situ measurement of isometric force

Isometric force of anterior (TA and EDL) muscles was measured in both legs in situ 3 d after ECC, as previously described [18]. Isometric contractions were elicited by peroneal nerve stimulation at 20 and 80 Hz. After the measurement, the TA muscles were excised from both legs. EDL muscles were not used in experiment 2 because large amounts of muscle samples are needed for analysis of nitrite/nitrate (NO_x) concentration.

NO_x concentration

Muscle NO_x levels were determined spectrophotometrically [18]. TA muscles were pulverized under liquid nitrogen and vortexed with 6 vol (vol mass-) of ice-cold phsophate0buffered saline (pH 7.4) containing 10 mM *N*-ethylmaleimide and 2.5 mM EDTA. The mixture was then centrifuged at 10 000*g*, and the resultant supernatant was mixed with Griess reagent. Absorbance at 540 nm was read after incubation at 37°C for 30 min.

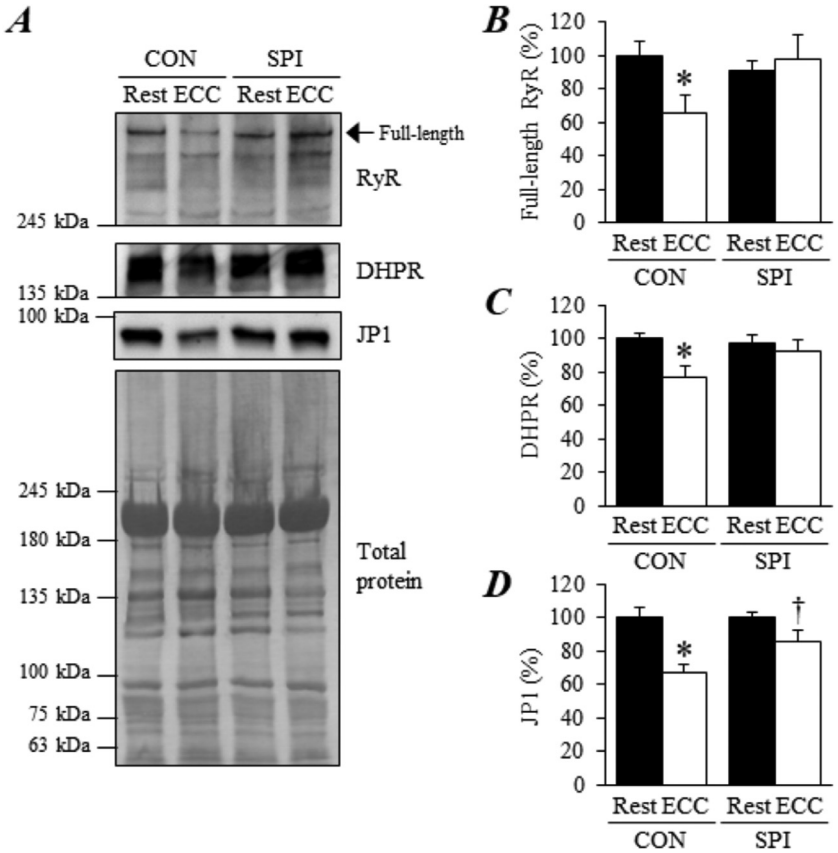

Fig. 2. Effect of SPI ingestion and ECC on levels of Ca^{2+}-regulatory proteins. For the protocols of SPI ingestion and ECC, see legend Figure 1. (A) Immunoblot analyses of RyR, DHPR, and JP1 in extensor digitorum longus muscles. For RyR, in addition to full-length proteins (arrow), faster migrating bands (degraded proteins) were observed. Position of molecular weight is indicated on the left. (B), (C), and (D), means ± SEM (n = 6 for each muscle) of the levels of full-length RyR, DHPR, and JP1, respectively. The results are expressed as percentages of the values observed in rested muscles from CON rats. Ca^{2+}, calcium ion; CON, control; DHPR, dihydropyridine receptor; ECC, eccentric contraction; JP1, junctophilin-1; RyR, ryanodine receptor; SPI, soy protein isolate. *$P < 0.05$ versus rested muscles. †$P < 0.05$ versus ECC muscles from CON rats.

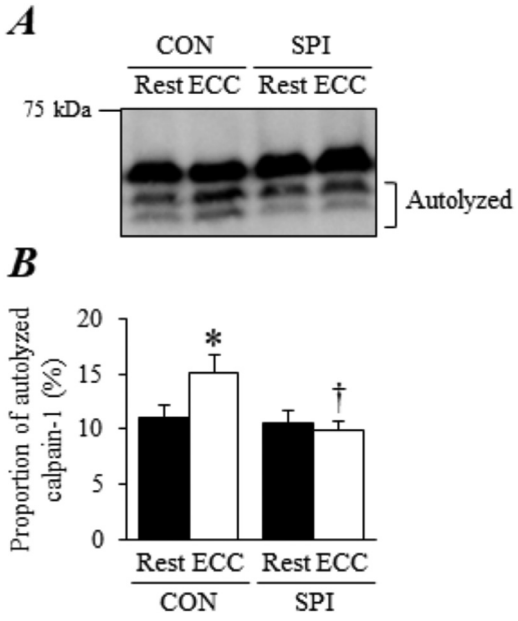

Fig. 3. Effect of SPI ingestion and ECC on autolysis of calpain-1. For the protocols of SPI ingestion and ECC, see legend of Figure 1. (A) Immunoblot analyses of calpain-1 in extensor digitorum longus muscles. A maximum of three bands were discerned. Of these, the upper band and the lower two bands represent full-length and autolyzed forms of calpain-1, respectively. Position of molecular weight is indicated on the left. (B) means ± SEM (n = 6 for each muscle) of the proportion of autolyzed calpain-1. Levels of the autolyzed form are expressed as percentages of total calpain-1. CON, control; ECC, eccentric contraction; SPI, soy protein isolate. $^{*}P < 0.05$ versus rested muscles. $^{\dagger}P < 0.05$ versus ECC muscles from CON rats.

Statistical analysis

Data are presented as means ± SEM. The effects of SPI and L-NAME ingestion and ECC were investigated using two-way analysis of variance. When significant differences were detected, the Holm-Šidák post hoc test was performed. $P < 0.05$ was considered statistically significant.

Results

Experiment 1

Force output

For 28 d, when the rats were fed distinct diets (a 20% casein or a 20% SPI diet), no significant differences in body weight were observed between CON and SPI rats (data not shown). In a preliminary experiment, we found that, immediately after ECC, tetanic forces at 20 and 80 Hz decreased in ECC muscles to ~40% and ~50% of those in rested muscles, respectively, in both rat groups.

Three days after ECC, the forces were partially restored in CON rats but remained depressed. The forces at 20 and 80 Hz in ECC muscles amounted to 69% and 83% of those in rested muscles, respectively (Fig. 1A, B). As a result of these changes, the ratio of force at 20 Hz to that at 80 Hz (20/80 Hz ratio) declined in ECC muscles to 74% of that in rested muscles (Fig. 1C). On the other hand, there were no significant differences in forces at 20 and 80 Hz between rested and ECC muscles in SPI rats, indicating that recovery to resting levels was attained for both 20 and 80 Hz. The forces at 20 Hz and the 20/80 Hz ratio, but not the forces at 80 Hz, differed significantly between ECC muscles of CON and SPI rats.

Levels of Ca^{2+}-regulatory proteins

In agreement with previous findings [5,18], degraded RyR proteins that migrated faster than full-length proteins were observed (Fig. 2A). In CON rats, the levels of full-length RyR, DHPR, and JP1 decreased in ECC muscles to 66%, 77%, and 67% of those in rested muscles, respectively (Fig. 2 B–D). It is widely accepted that the reduced amounts of these proteins occur because of increased proteolysis [5,8,18]. SPI ingestion caused complete inhibition of ECC-induced proteolysis; in SPI rats, there were no significant differences in the RyR, DHPR, or JP1 levels between rested and ECC muscles. The JP1, but not the RyR and DHPR levels, differed significantly between ECC muscles of CON and SPI rats.

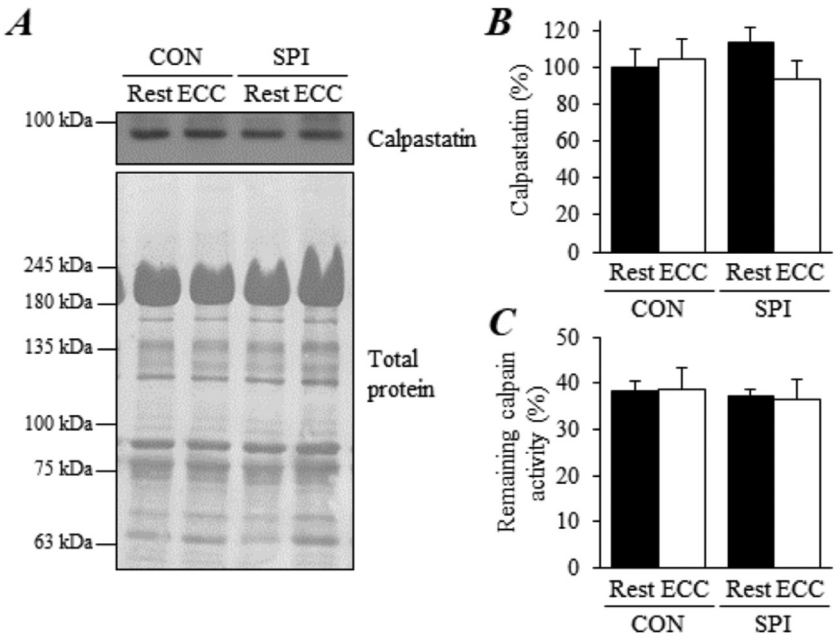

Fig. 4. Effect of SPI ingestion and ECC on levels and activity of calpastatin. For the protocols of SPI ingestion and ECC, see legend of Figure 1. (A) Immunoblot analyses of calpastatin in extensor digitorum longus muscles. Position of molecular weight is indicated on the left. (B) Means ± SEM (n = 6 for each muscle) of calpastatin levels. The results are expressed as percentages of values observed in rested muscles from CON rats. (C) Means ± SEM (n = 6 for each muscle) of the calpain-inhibitory activity of calpastatin. CON, control; ECC, eccentric contraction; SPI, soy protein isolate.

Levels of autolyzed calpain-1

It has been shown that, when calpain-1 is autolyzed, the Ca^{2+} concentration required for its activation decreases from ~2.5-50 to 0.5-2 μM [27] and that calpain-1 plays a central role in ECC-related proteolysis [5,6,10]. Given these findings, we investigated the levels of autolyzed calpain-1. Small amounts of calpain-1 (~11%) were present in autolyzed forms in rested EDL muscles (Fig. 3). In CON rats, the levels of autolyzed calpain-1 increased in ECC muscles to 136% of those in rested muscles, whereas ECC-related increases in autolysis were not observed in SPI rats.

Levels of calpastatin

SPI ingestion has been demonstrated to result in activation of calpastatin [22]. To examine whether calpastatin activation is involved in inhibited autolysis of calpain-1 observed in ECC muscles of SPI rats, we examined the levels and activity of calpastatin. Immunoblotting and fluorometric analyses indicated that neither SPI ingestion nor ECC had any obvious effect (Fig. 4).

Experiment 2

Force output

In CON rats, tetanic forces at 20 and 80 Hz decreased in ECC muscles to 59% and 84% of those in rested muscles, respectively (Fig. 5A, B). As a consequence, the 20/80 Hz ratio significantly declined in ECC muscles (Fig. 5C). SPI ingestion resulted in complete or partial restoration of force production after ECC. In SPI rats, there were no significant differences in the force at 80 Hz between rested and ECC muscles (Fig. 5B). The force at 20 Hz in ECC muscles remained low (87% of rested muscle levels) but was significantly higher than that in ECC muscles from CON rats (Fig. 5A). Co-ingestion of L-NAME with SPI negated the effect of SPI on force recovery; the forces at 20 and 80 Hz approximated between ECC muscles from CON and SPI + L-NAME rats.

NO_x levels

A previous study revealed that NO_x levels were capable of being used as an indicator of the levels of endogenously produced NO [18]. To ascertain whether faster recovery of force output after ECC with SPI ingestion relates to NO, the effects of L-NAME ingestion on the NO_x levels were investigated. In CON rats, the NOx levels tended to be higher in ECC than in rested muscles ($P = 0.056$), although the difference did not reach significance (Fig. 6). In SPI rats, the NO_x levels in rested and ECC muscles amounted to 209% and 257%, respectively, of that in rested muscles from CON rats. Co-ingestion of L-NAME with SPI completely inhibited SPI-induced increases in the NO_x levels. In SPI + L-NAME rats, the levels in rested and ECC muscles was similar to that in rested muscles from CON rats.

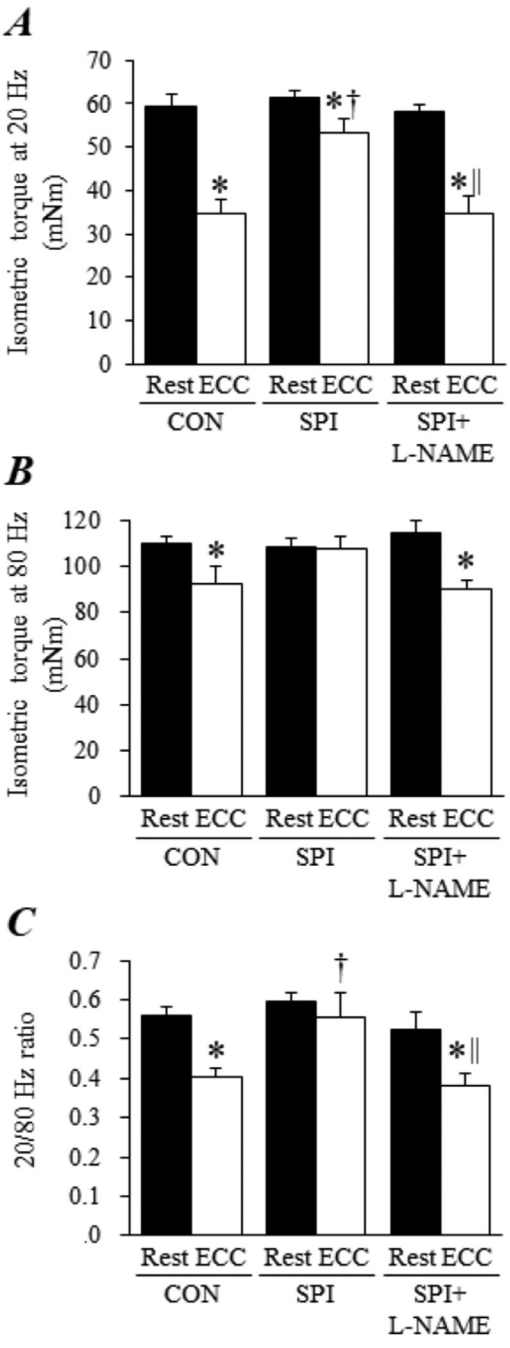

Fig. 5. Effects of SPI and L-NAME ingestion and eccentric contraction (ECC) on isometric force at 20 Hz (A) and 80 Hz (B) and force ratio (**C**). For the protocol of SPI ingestion and ECC, see legend of Figure 1. Some of SPI rats (SPI + L-NAME rats) were given water containing L-NAME, an inhibitor of NO synthase, for 3 d after ECC. Three days after ECC, in situ tetanic contraction of anterior crural muscles was evoked by peroneal nerve stimulation at 20 Hz and 80 Hz, and the ratio of force at 20 Hz to that at 80 Hz (20/80 Hz ratio) was calculated. Force production is expressed in milli-Newton-meters (mNm). Values represent means ± SEM (n = 5 for each muscle). CON, control; ECC, eccentric contraction; L-NAME, N^G-nitro-L-arginine-methyl ester; SPI, soy protein isolate. $^*P < 0.05$ versus rested muscles. $^†P < 0.05$ versus ECC muscles from CON rats. $^‡P < 0.05$, versus ECC muscles from SPI rats.

Discussion

The major findings in this study were that, in muscles undergoing ECC, SPI ingestion for 4 wk was responsible for a promotion of force recovery and inhibited calpain-1 autolysis and proteolysis of Ca^{2+}-regulatory proteins, as well as that L-NAME, an NOS inhibitor, attenuated ECC-related increases in NO_x levels and negated the force recovery effects of SPI.

To date, the effect of SPI ingestion on the proteolytic function of calpain was only examined in one study by Nikawa et al. [22]. They showed that SPI ingestion accounted for inhibition of calpain-1 activation, which is in accord with the results presented here, but that the inhibition was caused by increased activity of calpastatin, an endogenous inhibitor of calpain. Although we used the same protocols for SPI ingestion as Nikawa et al., our results regarding calpastatin are in disagreement with theirs. Although it is possible that subtle differences in SPI levels ingested by rats could yield different results, the reason for the conflicting results between studies remains unclear.

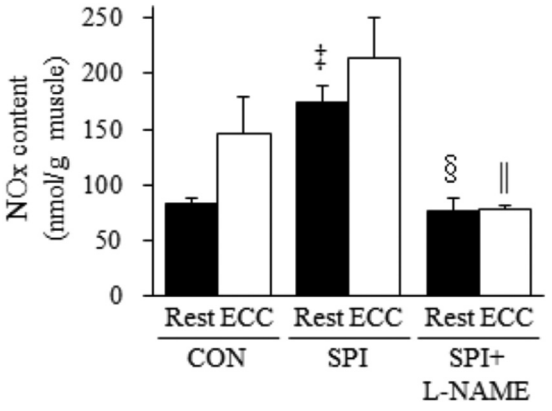

Fig. 6. Effects of SPI and L-NAME ingestion and ECC on NO_x levels. For the protocols of SPI and L-NAME ingestion and ECC, see legend of Figures 1 and 5. Values represent means ± SEM (n = 5 for each muscle). CON, control; ECC, eccentric contraction; L-NAME, N^G-nitro-L-arginine-methyl ester; NO_x, nitrite/nitrate; SPI, soy protein isolate. $^{*‡}P < 0.05$ versus rested muscles from CON rats. † $^{§}P < 0.05$ versus rested muscles from SPI rats. $^{‡‖}P < 0.05$, versus ECC muscles from SPI rats.

Our previous investigation [18] indicated that ARG ingestion results in inhibition of ECC-induced activation of calpain-1 through the following processes:

1 ARG ingestion brings about increases in NO generated by NOS in quiescent muscles.
2 Increased NO levels lead to *S*-nitrosylation of calpain-1, which reduces its proteolytic function.
3 In muscles subjected to ECC, *S*-nitrosylation evokes inhibition of calpain-1 activation that occurs as a consequence of the entry of extracellular Ca^{2+}.

It seems likely that these processes also occur in muscles from rats fed the SPI diet, based on the present results showing that SPI ingestion attenuated ECC-induced autolysis of calpain-1; ingestion of L-NAME negated the effect of SPI on force recovery and inhibited SPI-elicited increases in NO_x levels. It is of interest that, despite elevated NO_x levels, activation of calpain-1 was not mitigated in ECC muscles from CON rats (Fig. 3). The likely explanation for this is that, in these muscles, NO may interact with superoxide to form peroxynitrite, owing to which calpain-1 is less *S*-nitrosylated (for details, see discussion in Kanzaki et al. [18]).

ECC is widely known to elicit not only long-lasting force deficit, but also a greater loss of force at low- compared to high-stimulation frequencies [18,28], which is called prolonged low-frequency force depression (PLFFD) [29]. Our results regarding force production are consistent with the well-accepted view. It is recognized that PLFFD is ascribable to decreased myofibrillar Ca^{2+} sensitivity and/or reduced Ca^{2+} release from the sarcoplasmic reticulum (SR). A very recent study [18] indicated that reduced SR Ca^{2+} release, but not decreased myofibrillar Ca^{2+} sensitivity, was implicated in PLFFD that occurs with ECC. RyR, DHPR, and JP investigated in the present study are major proteins that regulate SR Ca^{2+} release and are substrates for calpain [5,18,30]. It is quite plausible, therefore, that calpain-mediated proteolysis of these proteins observed in ECC muscles from CON rats (Fig. 2) is responsible, at least partly, for reduced SR Ca^{2+} release, which in turn causes PLFFD, and that SPI-related inhibition of the proteolysis contributes to faster recovery of force production.

Conclusions

To our knowledge, very little information exists on the effective strategies toward overcoming muscle damage and subsequent depression in muscle performance resulting from ECC. The present investigation provides evidence that SPI ingestion can lessen ECC-elicited force deficit and proteolysis of Ca^{2+}-regulatory proteins, which is caused by inhibited activation of calpain-1 via increased NO production. Considering that, in muscles undergoing ECC, SPI-induced changes resemble those of ARG [18,19] and that SPI contains large amounts of ARG [21], the beneficial effects of SPI ingestion would thus stem from the increased intake of ARG. In further studies, it would be beneficial to investigate whether ingestion of a soy protein–rich diet is also an effective method for recovery of muscle performance after ECC in humans.

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