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Induction of muscle weakness by local inflammation: an experimental animal model

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Abstract

Objective and design—The objective of this study was to characterize the response of skeletal muscle to a localized inflammation induced by the inflammatory agent casein.

Methods—An inflammatory agent, casein, was injected into the right hindlimb and saline was injected into the left hindlimb of normal adult mice, once daily for six consecutive days. Inflammatory response was monitored by immunohistochemical labeling of leukocytes. Muscle protein levels were determined by electrophoresis and muscle function was determined by isometric force measurements.

Results—Local inflammation was induced by casein in association with the accumulation of extensive neutrophils and macrophages in the soleus muscle. This local inflammation resulted in a shift in myosin heavy chain (MHC) isoform expression and a significant reduction in total MHC concentration in the soleus. Maximal twitch and tetanic forces were significantly reduced in the inflamed soleus. Contractile function in soleus was fully restored after two weeks of recovery, along with the restoration of protein concentration and the disappearance of inflammatory cells.

Conclusion—This study establishes a unique and robust model in which mechanisms of local inflammation induced muscle protein degradation, reduction of contractile force, and subsequent recovery from this condition can be further studied.

Keywords

Myosin heavy chain; Isoform; Skeletal muscle; Force

Introduction

Inflammation, as an underlying cause of muscle weakness, has been suggested in various diseases, especially in critically ill patients. For example, muscle wasting invariably occurs in cachexia induced by bacterial sepsis [1], autoimmune disorders [2], cancer [3], and AIDS [4]. The notion that inflammatory processes are critical for the induction of muscle weakness in these conditions is supported by strong evidence demonstrating that inflammatory cytokines TNF α [5], IL-1 [6], and/or IL-6 [7,8] mediate muscle wasting during cachexia. In other pathological conditions, a weaker relationship between inflammation and reduced muscle function has been found. Thus, in inflammatory myopathies (e. g., dermatomyositis, polymyositis, and inclusion-body myositis), loss of muscle function has been associated with infiltration of inflammatory cells into muscle [9], with or without clear evidence of underlying causes for the inflammation. The critical mediators of inflammatory myopathies have not been

identified, although inflammatory cytokines have been implicated [10], [11], [12]. In aging [13], muscle disuse [14], fibromyalgia [15], and chronic fatigue syndrome, induction of muscle weakness by pro-inflammatory cytokines has been suggested, but a clear association has not yet been confirmed.

Mechanistically, severe inflammation, as occurs in sepsis, is coupled with a significant loss of muscle mass [16–18]. The widely accepted paradigm is that inflammation causes a change in the balance of muscle protein synthesis and degradation, tipping the scale toward protein degradation. Indeed, increased levels of pro-inflammatory cytokines have been inversely correlated with levels of the protein synthesis stimulator, IGF-1, during sepsis [19]. Blockade of inflammatory cytokines has been shown to be effective in preventing the alteration in sepsis-induced IGF levels [16,18], suggesting inflammatory cytokines can repress this important protein synthesis signaling pathway. In addition, increased levels of inflammatory cytokines, concomitant with decreased activity of the IGF system, have been observed in aging [20], chronic inflammation [20], inflammatory bowel disease [21], and ischemic heart disease [22], conditions also associated with muscle protein loss. In addition, pro-inflammatory cytokines have also been shown to promote protein degradation [23].

The direct effects of inflammation on muscle function *in-vivo* have not been studied. In a complex model of systemic inflammation-induced cachexia, besides the direct interaction between inflammatory cytokines and muscle tissue, many indirect effects of the host responses could be important. For example, the induction of stress hormones such as glucocorticoids could significantly alter muscle mass [24]. It has also been shown that sepsis induces a decrease in IGF-1 level in the circulation, which does not depend upon the action of inflammatory cytokines [25]. TNF α , however, has been shown to act directly in muscle to decrease muscle specific expression of IGF-1 [26]. Therefore, separating the systemic effects and the local effects of inflammation could help define the role of inflammatory cytokines in directly causing muscle weakness. This, in turn, could provide valuable insight into developing more effective anti-inflammatory therapies for humans, in general, and especially for sports medicine. Thus, our objective was to characterize the response of skeletal muscle to a localized inflammation induced by the inflammatory agent casein. Casein is an effective chemotactic agent and has been used in previous studies to induce inflammation by promoting infiltration of neutrophils into various sites *in-vivo* [27,28].

Materials and methods

Animals and experimental treatments

FVB mice (Jackson Laboratory, Bar Harbor, ME), 2–3 months old and weighing 25–30g, were used in this study. All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of The Ohio State University and conformed to the NIH Guide for the Care and Use of Laboratory Animals. The mice were given daily intramuscular injections of casein (10 % (w/v), 100 μ l) in their right hindlimb for six consecutive days. Injections were made into the caudal region of the hindlimbs in a caudocranial direction through the gastrocnemius muscle. Ten grams of casein (ICN Biomedicals, Aurora, OH) were added to 80 ml of 50 mM sodium bicarbonate (Sigma, St. Louis, MO). Water was then added to bring the total volume to 100 ml. The mixture was stirred and warmed to 65 °C until the casein was completely dissolved. The solution was filtered through coarse filter paper, aliquoted, and stored at –20 °C until injected. The left hindlimb was injected with saline (100 μ l) each day. The animals were anesthetized with isoflurane before each injection. Two mice were used to determine the area of casein delivery. The right hindlimb of one mouse was injected 0.3 % methylene blue (100 μ l of 0.3 % methylene blue) and the other mouse was injected methylene blue in combination with casein (20 μ l of 0.3 % methylene blue and 80 μ l of 10 % casein solution). The mice were euthanized several minutes later and the soleus, plantaris and

gastrocnemius muscles were dissected as an intact complex. Images of the muscle complex were obtained from several angles.

In the first group, the mice were euthanized one day after the last of six daily injections to examine the effects of local inflammation on muscle function and protein expression. In two other groups of animals, the same protocol was used for administering casein and saline into the hindlimbs. The animals in these two groups were allowed to recover for either one week or two weeks to study the recovery phase of muscle damage induced by casein injections.

Muscle isometric force measurements

Contractile properties of isolated mouse soleus muscles were determined as described previously [29]. Force measurements were limited to the soleus muscle because the histological results from the methylene blue-injected mice indicated that casein was highly localized around this muscle and the gastrocnemius and plantaris muscle widths are regarded as being too large for adequately rapid oxygen diffusion from the bathing solution. Briefly, mice were euthanized and both hindlimbs, with skin removed, were placed in a petri dish containing continuously oxygenated (95 % O₂/5 % CO₂) Krebs-Ringer's solution (137 mM NaCl, 5 mM KCl, 13 mM NaHCO₃, 1.8 mM KH₂PO₄, 2 mM CaCl₂, 11 mM glucose, 1 mM MgSO₄, and 0.025 mM tubocurarine chloride). All chemicals were purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO). Both the right and left soleus muscles were carefully dissected. The proximal and the distal tendons of one soleus were glued (Loctite Superglue, Henkel Consumer Adhesives, Inc., Avon, OH) to two rigid metal wires. The muscle was connected, with one of the wires, to a fixed post in the tissue chamber of a model 800A *in-vitro* test apparatus (Aurora Scientific, Inc., Aurora, Canada) and to a 300B dual-mode muscle lever system (Aurora). The lever system was used in the length-control (isometric) mode and the force output from this system was analyzed to measure muscle contractile properties. Force records were captured using DASyLab software (DASyLab 5.5, IO Tech, Cleveland, OH) at a sampling rate of 20 kHz. The other muscle was kept in the oxygenated Krebs-Ringers solution in the same petri dish at room temperature while measurements were made on the first muscle. All of the measurements of contractile properties were made at ambient temperature. The mean temperature of the chamber bath was 23.8 °C. Results from an initial set of experiments indicated that the order in which the muscles were studied (i. e., casein-treated, then saline-treated, or vice versa) from a given mouse did not affect the results. Nevertheless, the order of studying the muscles was alternated for each mouse. The output of the stimulator (Grass Medical Instruments Co., model SD9; typically set at 100 volts, with maximal output of 30 watts) and the length of the muscle were adjusted until maximal force was generated during an initial series of twitches. The muscle was then stimulated (each stimulus being 2 ms in duration, using a supramaximal voltage) to elicit five twitches. The peak twitch force (P_T), time to peak twitch force (T_P), and the time from the peak of the twitch to one-half relaxation (T_{1/2R}) during each twitch were recorded. The mean value of each of these parameters from the five twitches was calculated for each muscle. The tetanic stimulus was applied at 20 Hz (determined during preliminary experiments to elicit maximal force) for 0.4–0.5 sec. The maximal, steady tetanic force (P_O) and the time for one-half relaxation, from the instant that the stimulus train ended, were determined for each of five tetani, spaced five minutes apart. The mean values of both parameters from the five tetani were calculated. Results are presented as mean ± standard error of the mean (SEM) for the twitches and tetani of all of the muscles for each of the groups. The length of the muscle at which contractile properties were measured was determined using a small ruler submersed in the chamber. Muscle mass was determined after the muscle was removed from the chamber following quick removal of the tendons. Peak twitch and tetanic forces were normalized with muscle cross-sectional area, the latter estimated as mass/length, assuming a tissue density of 1.00 gm/cm³. The muscles were frozen until analysis with gel electrophoresis, and immunohistochemical and histological analyses.

Gel electrophoresis

The myosin heavy chain (MHC) isoform composition of homogenates of muscles was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as in Bicer and Reiser [30]. The same equivalent wet muscle mass (10.0 μ g) was loaded in each gel lane so that the amount of MHC in each sample, relative to others, could be determined by scanning densitometry. Comparisons of total MHC protein amounts were limited to samples run on the same gel, due to potential differences in stain intensity between gels. The relative amount (i. e., percent of total MHC) of each MHC isoform in individual samples, was also determined by scanning densitometry. The same muscle homogenates were also run on 12 % acrylamide gels to examine potential differences in the expression of other, lower molecular weight proteins between casein-treated, saline-treated, and recovery muscles. Myosin light chain bands on gels were identified using a myosin extraction protocol, as described in Bicer and Reiser [31].

Immunohistochemical staining of infiltrating immune cells

Muscle tissue was collected, immediately following euthanasia, from a separate group of animals subjected to the same casein and saline injection protocol. Tissue samples were cut in half. One half was fixed in 10 % formalin for H&E staining. The other half was stored in -80° C until being cut into 10 μ m-thick sections on a cryostat. Tissue sections were air-dried and fixed in a mixture of 75 % acetone and 25 % ethanol for 5 min. Glucose oxidase and sodium azide were used to reduce background interference. Sections were incubated with Ly6G, a neutrophil-specific antibody, and F4/80, a macrophage specific antibody (BD Bio-sciences, Franklin Lakes, NJ), using a 1:100 dilution. Biotinylated rabbit anti-mouse IgG (Vector Laboratories, Burlingame, CA) was used as the secondary antibody. The reactions were visualized with streptavidin/ HRP (1:500/1:2,500).

Mass spectrometry

Compared to the saline-injected soleus, the casein-injected soleus had three protein bands with markedly increased abundance, as observed with SDS-PAGE. Casein-treated soleus homogenates were heavily loaded onto a low molecular weight gel (12 % acrylamide). The gel was stained with Coomassie Blue and, after de-staining, the three bands were excised and stored in 5 % acetic acid until they were processed for protein identification, as described in Bergin et al. [32]. Mass spectrometry was performed at The Ohio State University Mass Spectrometry and Proteomics Facility.

Statistical analysis

Casein and saline treated groups were compared using Student's t-test. All of the data are reported as mean and SEM. P values less than 0.05 were accepted as indicating a significant difference between means.

Results

Physical characteristics

All animals exhibited swelling in the casein-injected hindlimbs after six days of injections (Figure 1). Casein-injected soleus mass was significantly greater than the saline-injected soleus mass (17.1 ± 1.0 mg and 8.2 ± 0.7 mg, respectively). The mean mass of the soleus in non-injected control mice was 7.4 ± 0.3 mg. Saline-injected soleus mass did not differ from control soleus mass.

Histological and immunohistochemical analysis

Histological examination of the gastrocnemius, plantaris, and soleus muscles of two mice that were injected with methylene blue only (Figure 2A, B) or injected simultaneously with casein and methylene blue (Figure 2C, D), using the same injection protocol as for the rest of the study, revealed that the casein was highly concentrated in the region surrounding the soleus muscle. Histological sections of soleus muscle after six consecutive days of casein injection are shown in Figure 3. An H&E stained section of non-injected control soleus is shown in Figure 3A. H&E staining revealed that saline injections for six consecutive days (Figure 3B) did not induce inflammation. Six days after casein injections, the presence of leukocytes in the muscle was evident (Figure 3, C). Neutrophil- and macrophage-specific staining showed the presence of neutrophils (Figure 3, F) and macrophages (Figure 3, I) in the casein-injected side.

Protein and mass spectrometry analysis

We examined MHC isoform expression patterns after six days of casein or saline injections (Figure 4, results from high-molecular weight gel electrophoresis). The densitometry results are listed in Table 1. The relative amounts of fast-type MHC-IIA and MHC-IID isoforms were decreased and the relative amount of slow-type MHC-I isoform was increased in the soleus muscles in the casein-injected hindlimbs. The total amount of MHC in the soleus after the casein injections was reduced by 67 % from the control level (contralateral saline soleus).

Three protein bands with molecular weight lower than MHC, were observed to be more abundant in muscles on the casein-injected side (Figure 5). These bands were identified by mass spectrometry (Table 2) as S100-A8, with known neutrophil chemotactic activity, fatty acid-binding protein and alpha-enolase, a multifunctional enzyme.

Force generation in inflamed soleus muscle

To determine whether muscle weakness is associated with inflammation, we measured peak isometric twitch and tetanic forces in casein-injected and saline-injected soleus muscles. Figure 6 shows a typical twitch (A) and tetanus (B) in a control muscle. Figure 7 shows the twitch (A) and tetanic (B) forces, normalized with muscle cross-sectional area, of the soleus from casein-injected hindlimbs and saline-injected, contralateral hindlimbs, as well as from control, non-injected mice. Casein injection induced significant reductions in normalized twitch and tetanic forces to ~25 % of control values. Absolute (i. e., not normalized with muscle cross-sectional area) mean twitch force (16.5 ± 2.9 mN and 34.1 ± 0.9 mN, casein and saline, respectively) and mean tetanic force (74.2 ± 16.5 mN and 167.6 mN, casein and saline, respectively) were also significantly lower. The absolute twitch and tetanic forces generated by control soleus from non-injected limbs were 33.1 ± 1.4 mN and 127.4 ± 5.9 mN, respectively. The time to peak twitch force and the time to one-half relaxation of the twitch did not differ between groups. The time to one-half relaxation at the end of the tetanus was longer in the soleus muscles from the casein-injected hindlimbs (0.32 ± 0.04 sec), compared to the saline-injected hindlimbs (0.22 ± 0.01 sec).

The pH of the injected casein solution was 6.3 at 37 °C, whereas the pH of the saline solution used on the control side was 7.2 at 37 °C. Therefore, a series of experiments was conducted to test whether the lower pH of the casein solution could, by itself, have caused the observed differences in force generation between the casein and saline injected sides. Three mice were injected on six consecutive days with saline. The pH of the saline was not adjusted (7.2 at 37 °C) on one side and, on the other side, the pH was titrated to 6.3 at 37 °C with HCl. Peak twitch force was 49.8 ± 2.6 kN/m² on the pH 6.3 side and 45.7 ± 1.2 kN/m² on the pH 7.2 side. Peak tetanic forces were 181.6 ± 10.2 and 189.0 ± 14.6 kN/m² on the two sides, respectively. Neither of the differences was significantly different.

Soleus force generation after recovery

To study recovery from inflammation in this model, we carried out the same six-day casein-injection protocol, followed with one week or two weeks without injections. Figure 8 shows the results of force measurements after recovery. The twitch force (A) and tetanic force (B) recovered to ~80 % of the control levels after one week of recovery (Figure 8), but were still significantly lower than control values. After two weeks of recovery, casein injected muscles were no longer significantly different from the saline injected muscles with respect to peak twitch (Figure 8, C) or tetanic (Figure 8, D) forces, indicating complete recovery by this time.

Myosin and histological analyses after recovery

After one week of recovery, the relative levels of MHC-I, -IIA, and -IID isoforms in the soleus on the casein-injected side were no longer different from saline-injected contralateral soleus (Table 1). However, the amount of the physiologically fastest isoform, MHC-IIB, was increased significantly (Table 1; Figure 9, A) after one week of recovery. After two weeks of recovery, the relative amounts of all of the MHC isoforms in the soleus muscles on the casein-injected side were not different from those on the saline-injected side (Table 1; Figure 9, B).

S100A8, ENOA, and E-FABP, proteins that were induced in casein injected soleus, diminished after either 1 or 2 weeks of recovery (Figure 5).

Evidence of recovery was also observed in tissue sections stained with H&E (Figure 10). Neutrophil numbers appeared to be similar to controls after one week (Figure 10, A) and two weeks (Figure 10, B), following the final casein injection.

Discussion

The results of the present study show that intramuscular injection of casein induces localized inflammation at the site of injection. Previous studies have shown that this inflammatory agent does not cause systemic production of cytokines, but induces high level production of inflammatory cytokines locally [27,28]. Interestingly, a unique feature observed in the present study is that muscle protein degradation is found in inflamed muscle without overt signs of muscle damage or injury. Other studies have demonstrated a role of leukocytes in causing further muscle damage when the muscle tissue has been physically injured [33]. Therefore, the present model could be valuable for studying mechanisms of altered muscle function in association with local inflammation, per se, and subsequent recovery from this condition.

After the cessation of casein injections, muscle function largely recovered in one week, and completely recovered in two weeks. Thus, these results establish a muscle weakening and recovery model in which local inflammation induced highly reproducible and large, but reversible reductions in muscle function. This model should be useful for the analysis of molecular mechanisms through which local inflammation causes muscle weakness and factors that promote recovery, possibly involving increased muscle protein synthesis.

Although inflammation has been suspected to be a major contributing factor in the pathogenesis of muscle degeneration, a systematic and definitive analysis of the role of inflammation has not been reported. In inflammatory myositis, the underlining cause of inflammation could be variable. Bacterial [34], viral [35] [36], parasitic [37] [38], and allergic [39] origins have all been documented. Because the immune systems react to these different types of challenges differently, it would be difficult to establish a single model to study all types of inflammatory myopathy. The current study focuses on establishing an experimental animal model to study muscle weakness induced by the injection of a simple inflammatory agent, casein. It is known that this agent induces a strong production of local pro-inflammatory cytokines and acute phase proteins without inducing circulating cytokines [27,28]. The present results show that casein

injection induces accumulation of neutrophils and macrophages at the site of injection, demonstrating the induction of innate inflammatory responses in this model. We did not choose other more commonly used inflammatory agents such as LPS, because LPS injection is known to produce systemic effects, e. g., release of glucocorticoids and circulating cytokines [40], such that the muscle damage may be induced due to these factors released from multiple distal systems. That the present model causes restricted muscle weakness is corroborated by the fact that significant muscle protein degradation was induced after casein injection only on the casein side, without muscle weakness on the contralateral saline-injected side. Therefore, this model may allow precise analysis of muscle weakness induced by local inflammatory factors.

Examples of selective muscle damage have been reported in association with ischemia [41], eccentric contractions [42], and alcohol consumption [43]. In these cases, fast muscles, with predominantly type II fibers, are preferentially susceptible. In the present study, decreased amounts of MHC-IIA and of MHC-IID (normally expressed in fast fibers) and increased amount of MHC-I (slow fibers) were found in casein-injected soleus without evidence of regeneration and histological signs of muscle injury. The reduction in force generating ability examined in the soleus muscle appears to be correlated with the loss of protein integrity, especially loss of MHC. Significant reductions in twitch and tetanic forces in soleus muscle coincided with casein-induced protein, especially MHC, degradation in this muscle. After 1–2 weeks of recovery, the return of contractile function is associated with the restoration of MHC concentration in the soleus. Interestingly, embryonic and neonatal MHC isoforms, hallmarks of muscle regeneration [44,45], were not detected in any muscle after six days of casein injections or following one week or two weeks of recovery (Figure 9). These results suggest that, whereas muscle protein regeneration is a component of recovery in this model, activation of classical muscle regeneration pathways is not. Thus, the present study revealed a novel and subtle phenotype: inflammatory events occurring in the present model might have triggered signaling of increased protein degradation, signals to which the fast fibers may be more sensitive. This is different from overt fast fiber damage. Contraction induced muscle damage, e. g., results in significant histological signs of tissue injury [33]. It should also be noted that this local inflammation model causes significant edema which could also contribute to the reduced muscle contractile forces.

This study also revealed three proteins in inflamed muscle, S100, Alpha-enolase, and E-FABP. S100 proteins are pro-inflammatory cytokines that play an important role in the recruitment of leukocytes (reviewed in [46,47]). Our results show increased S100-A8 protein following casein injections and that S100-A8 was down-regulated during recovery. The kinetics of S100-A8 is in parallel with the changes in the number of infiltrating leukocytes. Therefore, the level of S100A8 could be a marker for the extent of local muscle inflammation.

Casein also induced increased expression of Alpha-enolase (Table 2), a multifunctional enzyme, known for its critical role in myogenesis [48]. It is up-regulated during denervation-reinnervation and regeneration of skeletal muscle [49]. It is possible that Alpha-enolase plays a role other than myogenesis in this model because evidence of regeneration is not observed.

The results of this study establish that casein can be used to create a model of localized muscle inflammation. The inflammatory response was very dynamic, recovering within one or two weeks following casein injections, and was multifactorial, involving a marked increase in the intramuscular monocyte populations and significant reductions in myosin protein content and force generating ability, as well as changes in myosin isoform expression. This model will be valuable to examine molecular and cellular mechanism of muscle inflammation, as well as the dynamics of recovery with possible therapeutic interventions.

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Fig. 1.

Top: Image of a representative mouse which had received casein and saline injections on each of the preceding six days. Bottom: muscles from the dorsal side of the hindlimbs from the same mouse (the gastrocnemius muscle is superior on both sides; Achilles tendon at bottom).

Fig. 2.

A: Lateral view of the soleus-plantaris-gastrocnemius complex from a mouse injected with methylene blue two minutes before euthanasia. The caudodorsal (curved) surface is on the left. B: Cranioventral surface of the same muscle complex. C and D: Cranioventral surface (rotated slightly in C to show the unstained lateral edge of the gastrocnemius) of the soleus-plantaris-gastrocnemius muscle complex from a mouse injected with methylene blue and casein two minutes before euthanasia. The border of the soleus muscle is indicated by the white dotted line.

Fig. 3.

Representative micro-photographs showing that casein injection induced localized inflammation. A, B, and C are H&E stained sections of muscle tissue near the site of injection. D, E, and F are neutrophil stained sections (Ly6G), G, H, and I are macrophage stained sections (F4/80). A, D, and G are the control non-injected animal; B, E, and H are from the saline-injection side; C, F, and I are from the casein injection side.

Fig. 4.
The myosin heavy chain (MHC) region of a gel showing the fast (MHC-IIA, and -IID) and slow (MHC-I) MHC isoform composition of soleus muscles after 6 days of casein (C) or saline (S) injections.

Fig. 5.

Low molecular weight gel onto which homogenates of soleus muscles from three mice injected once daily for six days, on one side with casein, and on the other side with saline, or following one week or two weeks of recovery, were loaded. Fast (F) and slow (S) single fibers from control muscle were also loaded (in the right side of the gel) to facilitate identification of the fast and slow isoforms of myosin light chains. The positions of proteins identified by mass spectrometry were marked on the left side of the gel. MHC, myosin heavy chain; Tm- α , Tm- β , alpha and beta isoforms of tropomyosin; MLC, myosin light chain; S, slow; F, fast; ENOA, alpha-enolase; E-FABP, epidermal type fatty acid-binding protein; S100A8, protein S100-A8. Note that ENOA migrated slightly slower than another protein that was present in all of the samples.

Fig. 6. Examples of an isometric twitch (A) and tetanus (B) in a control mouse soleus muscle. Parameters measured in this study: time to peak twitch force (T_p), amplitude of peak twitch force (P_t), amplitude of peak tetanic force (P_o), and time to one-half relaxation ($T_{1/2R}$, both twitch and tetanus).

Fig. 7.

Peak twitch (A) and tetanic (B) force, normalized with muscle cross-sectional area, in soleus muscles from control mice (non-treated) (n = 6), saline-injected hindlimbs (n = 6), and casein-injected hindlimbs (n = 6). Muscles were studied one day following six consecutive days of treatment. Controls were age-matched. Values are mean $\pm$ SEM. *Casein different from both control and saline.

Fig. 8. Peak twitch (A and C) and tetanic (B and D) force, normalized with muscle cross-sectional area, after one week (A and B) ($n = 3$) and two weeks (C and D) ($n = 3$) of recovery, following six consecutive days of casein or saline treatments. Mean $\pm$ SEM, *Casein different from saline.

Fig. 9.

The myosin heavy chain (MHC) region of gels showing the fast (MHC-IIA, -IID, and -IIB) and slow (MHC-I) isoforms of soleus muscles after one week (A) and two weeks (B) of recovery, following casein (C) or saline (S) injections. Abdominal skeletal muscle homogenates from one day old (P1) and seven day old (P7) pups were used as standards for embryonic MHC (MHC-Emb) and neonatal MHC (MHC-Neo) isoforms.

Fig. 10. Representative microphotographs show H&E staining of soleus muscle tissue from animals with one week of recovery (A) or two weeks of recovery (B) after casein injections.

Table 1

Relative levels (i. e., percent of total MHC) of soleus myosin heavy chain (MHC) isoforms after six days of casein injections without recovery, 1 week of recovery, and 2 weeks recovery.

	No recovery (n = 6)		1 week recovery (n = 3)		2 weeks recovery (n = 3)	
	Casein	Saline	Casein	Saline	Casein	Saline
MHC-IIA	31.3 ± 2.2 *	41.9 ± 0.9	34.2 ± 1.5	40.3 ± 2.0	35.7 ± 3.8	36.9 ± 3.0
MHC-IID	3.0 ± 0.9 *	10.2 ± 2.0	14.0 ± 2.9	14.7 ± 2.1	7.2 ± 2.2	11.8 ± 0.6
MHC-IIB	0.6 ± 0.4	0.4 ± 0.2	7.8 ± 2.4 *	0.1 ± 0.1	2.6 ± 1.0	2.2 ± 2.2
MHC-I	65.1 ± 2.6 *	47.2 ± 2.0	43.5 ± 3.6	44.9 ± 0.9	54.5 ± 4.7	49.2 ± 5.4

Asterisk indicates significant difference ($p < 0.05$) between casein-injected and saline-injected muscles. Values are mean ± SEM.

Table 2

Mass spectrometry-based identification of proteins with increased abundance in casein-injected soleus.

Protein identification	Accession	Database	Peptides matched	PBMS ^I	Mass (Da)
Alpha – enolase	P17182	SwissProt	28	803	47453
Fatty acid-binding protein	Q05816	SwissProt	4	247	15470
Protein S100-A8	P27005	SwissProt	20	247	10345

^IProbability-based MOWSE score.