

Muscle impairment occurs rapidly and precedes inflammatory cell accumulation after mechanical loading

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Frenette, Jérôme, Matthieu St-Pierre, Claude H. Côté, Eleni Mylona, and Frank X. Pizza. Muscle impairment occurs rapidly and precedes inflammatory cell accumulation after mechanical loading. *Am J Physiol Regulatory Integrative Comp Physiol* 282: R351–R357, 2002; 10.1152/ajpregu.00189.2001.—Modified muscle use can result in muscle atrophy and impairment. We tested whether inflammatory cell concentrations correlate temporally with muscle impairment during modified loading periods. Rat hindlimbs were unloaded for 10 days followed by reloading. The density of neutrophils and ED1⁺ macrophages was significantly increased by 16.5- and 9.8-fold, respectively, after 1 day of reloading. ED2⁺ macrophage concentration was not significantly increased until 3 days of reloading. Maximal isometric tetanic tension (P_o ; N/cm²) decreased during hindlimb suspension (HS), which was followed by a second drop in P_o after 2 h of reloading. This latter loss in muscle force was uncoupled with the significant elevation in muscle inflammatory cell concentrations. Experiments where HS soleus muscles were incubated with caffeine revealed that at least 40% of the P_o decrement at 2 h could be associated with a loss of efficiency of the excitation-contraction (E-C) coupling process. These data suggest that an important mechanism for the early loss in force is the inability to activate the contractile machinery likely caused by a failure in the E-C coupling process during the reloading period.

rat; macrophage; neutrophil; inflammation; muscle injury

PREVIOUS INVESTIGATIONS HAVE established that hindlimb suspension (HS) induces a loss of myofibrillar proteins and muscle mass (31). This atrophy is accompanied by a loss in maximal absolute isometric tension (P_o). An exacerbation in muscle damage is apparently initiated by the increase in mechanical stress and/or inflammatory cell invasion during the phase of reloading (33). Morphological observations obtained in vitro and in vivo indicate that leukocytes can, indeed, accentuate muscle damage by releasing free radicals and/or proteolytic enzymes (3, 17). The possibility that invading inflammatory cells contribute to muscle injury in the model of HS is indirectly supported by the observation that significant elevations in muscle neutrophil and

macrophage concentrations occur at time points when ultrastructural signs of injury are exacerbated and fiber necrosis occurs (7, 21, 30, 33). However, the contribution of inflammatory cells to muscle injury has not been convincingly demonstrated in response to modified mechanical loading (33).

It has been clearly shown that atrophic muscles are much more susceptible to increase in mechanical stress than normal muscles. For example, muscle deconditioned by microgravity or HS followed by normal ambulation induces structural lesions that resemble those produced by eccentric contractions (23, 24, 32). The contribution of mechanical reloading to muscle injury has been further exemplified by two spaceflight experiments where the reentry on earth with high-G impact produced eight times more fiber damage than low-G impact (22, 23). These findings are also consistent with previous reports showing that skeletal muscle is more prone to injury when sustained eccentric contractions are performed after a period of immobilization or unloading (1, 32).

An important step to ascertain the specific contribution of inflammatory cells and mechanical loading to the muscle injury that occurs during reloading is to determine the temporal relationship between muscle inflammation and contractile function. The goal of this study was to test the hypothesis that the inflammatory cell accumulation temporally correlates with the level of muscle impairment seen when muscles are reloaded after a period of HS. Inflammatory cell accumulation, fiber necrosis, and contractile properties were evaluated immediately after HS and in animals experiencing muscle reloading for 2 h, 24 h, 3 days, 7 days, and 28 days. These time points were selected because they encompass the phases of preinflammation, extensive inflammation, and resolution of the inflammatory process. Because of the rapid decline in muscle function at 2 h of reloading, we thus decided to verify if impairment of the excitation-contraction (E-C) process was evident within the first 2 h of reloading. We show that muscle impairment and E-C coupling failure occur

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earlier than the well-documented time course of inflammatory cell accumulation, which suggests that E-C coupling plays a very important role in muscle force decrement following HS.

MATERIALS AND METHODS

Experimental protocol. Female Wistar rats weighing between 200 and 250 g at death were used for this study. Experimental animals were subjected to HS for 10 days, followed by reloading periods of 0 h ($n = 6$), 2 h ($n = 5$), 1 day ($n = 6$), 3 days ($n = 6$), 7 days ($n = 6$), or 28 days ($n = 6$), as described previously (7, 21). Six rats that did not experience HS and had normal cage activity were used as a control group. All animal care and handling procedures were approved by the Laval University Research Center Animal Care and Use Committee.

Measurement of mechanical properties. At the end of the experimental protocol, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and both soleus (Sol) muscles were carefully dissected and incubated in vitro in Krebs-Ringer bicarbonate buffer supplemented with glucose (2 mg/ml). The viability of the preparations was provided by a constant bubbling of carbogen into the solution that was maintained at 25°C. In vitro measurement of muscle contractility was performed as previously described (4, 6, 18). One tendon was attached to a rigid support at the bottom of the bath, and the other end was connected to an isometric force transducer (Grass FT-03) through a stainless steel hook. An initial period of 30 min was allowed for equilibration of the muscles and to adjust muscles to their optimal length, defined as the length at which maximal isometric twitch tension is produced. At the end of the equilibration period, one single twitch contraction was elicited and the following measurements were obtained: maximum twitch tension (P_t), time to peak tension (TPT), and one-half relaxation time ($RT_{1/2}$). After measurement of twitch parameters, muscles were stimulated for 1 s at frequencies of 50, 80, 100, and 120 Hz to determine P_o . After measurement of P_o , Sol muscles were weighed without their tendons to quantify muscle mass and to allow calculation of specific P_o (N/cm²). The value used for muscle density was 1.062 g/cm³ (16), and the ratio of fiber length to muscle length used was 0.62 (28).

Evaluation of E-C coupling integrity. In a separate set of experiments, contractile properties of Sol muscles were measured in ambulatory controls ($n = 5$) and animals suspended for 10 days and reloaded for 0 h ($n = 5$) or 2 h ($n = 5$). After measurement of contractile properties, the efficiency of E-C coupling was then assessed by adding caffeine (50 mM) to the Krebs-Ringer bicarbonate buffer. Caffeine can bypass all essential steps that lead to Ca²⁺ release by the sarcoplasmic reticulum (SR) (13) and directly act on the calcium release channels of the SR. The increment in passive tension induced by caffeine was continuously monitored, and the absolute force value was recorded every 2 min until it plateaued. A decline in P_o while caffeine-induced force is maintained indicates that the contractile machinery is still able to respond to calcium ions released in the cytosol and that the E-C coupling process is not leading to an adequate calcium release.

Tissue preparation. All Sol muscles dissected, except those incubated in caffeine, were stretched and maintained near resting length by stapling their extremities to balsa wood coated with a thin layer of OCT compound. The muscle was then frozen in melted isopentane cooled in liquid nitrogen and stored at -70°C. Sol muscles were sectioned at 10 μ m, adhered to slides coated with chromium potassium sulfate and gelatin, and stored at -20°C.

Immunohistochemistry. Sections were processed for immunohistochemistry with the following antibodies: 1) anti-ED1⁺ and anti-ED2⁺ (diluted 1:100; Bioproducts for Science, Indianapolis, IN), which recognize antigens specific for different subpopulations of macrophages, and 2) anti-His-48 that reacts with an antigen specific for granulocytes (diluted 1:25; BD Pharmingen, San Jose, CA). Neutrophils represent more than 90% of the circulating granulocytes, and they are typically the first population of cells to invade skeletal muscle (7). The sections were then washed in PBS and incubated with biotinylated anti-mouse (diluted 1:200; Vector Laboratories, Burlingame, CA) for 1 h. After the secondary antibody incubation, tissue samples were washed with PBS and incubated with horseradish peroxidase (1:1,000; Vector Laboratories). After three washes, the antibody-antigen complex was revealed by chromogenic development using peroxidase substrate kit AEC (Vector Laboratories). The concentration of inflammatory cells labeled with each antibody was measured in two sections in both Sol muscles and examined blindly by light microscopy using Nomarski optics. The number of labeled cells in each section was counted, and the total area of the section was determined and multiplied by its thickness to express the number of each cell type per cubic millimeter.

Statistical analysis. The mean of the right and left Sol muscles was used for statistical analyses. All data were analyzed by a one-way analysis of variance to test whether the variation among experimental groups was significant at $P < 0.05$. When a significant F ratio was obtained, post hoc multiple comparison testing was done with a Fisher's protected least-significant differences test to determine where specific differences had occurred.

RESULTS

Morphological and contractile properties. The values of muscle mass for Sol muscles decreased by more than 48% compared with control values following 10 days of suspension; this was paralleled by a significant decrease of 5% in fiber length (Table 1). Muscles reloaded for 0 h, 2 h, 1 day, and 3 days showed significant decreases in muscle mass compared with ambulatory controls. Muscle mass recovered and reached ambulatory control values after 7 days of reloading. Contractile properties also changed during HS, suggesting a conversion in phenotype from slow- to fast-twitch muscle fibers. TPT was reduced by 25% after 10 days of HS and returned to ambulatory control value after 3 days of reloading. A similar response was observed for $RT_{1/2}$, which was reduced by 37% after HS. $RT_{1/2}$ values were no longer significantly different from ambulatory controls after 7 days of reloading. A 62% reduction in absolute P_t and P_o was also observed compared with ambulatory controls after 10 days of HS. The diminution of P_t and P_o was consistent with the significant muscle atrophy induced by unloading. Animals reloaded for only 2 h experienced a second drop in P_t and P_o , whose value decreased by another 50% compared with reloaded muscles for 0 h (Table 1).

Quantification of inflammatory cells and invaded fibers. Our findings show that each inflammatory cell type accumulated progressively in Sol muscles during the reloading periods. The density of neutrophils and ED1⁺ macrophages was significantly increased by 16.5- and 9.8-fold, respectively, after 1 day of reloading

Table 1. Morphological and contractile properties of Sol muscles from CTR and animals subjected to 10 days of hindlimb suspension followed by different reloading periods

	L_f , mm	Muscle Mass, mg	$RT_{1/2}$, ms	TPT, ms	P_t , mN	P_o , mN	P_o , N/cm ²
CTR	14.9 ± 0.2†	99.6 ± 2.4†	99.7 ± 5.9†	69.4 ± 2.6†	273.4 ± 11.8†	1,269 ± 45†	20.3 ± 0.7†
0H	14.1 ± 0.2*	51.3 ± 1.3*	62.3 ± 4.2*	52.0 ± 2.4*	105.8 ± 7.8*	474 ± 27*	13.0 ± 0.7*
2H	13.2 ± 0.3*	50.3 ± 2.6*	60.1 ± 2.6*	50.4 ± 2.6*	51.9 ± 9.8*†	247 ± 34*†	7.6 ± 1.3*†
1D	14.6 ± 0.1	55.0 ± 4.0*	73.4 ± 2.4*†	61.9 ± 1.0*†	84.3 ± 1.1*	333 ± 52*	9.6 ± 0.7*†
3D	14.8 ± 0.1†	69.4 ± 2.0*	81.0 ± 5.8*†	70.9 ± 3.2†	113.7 ± 0.7*	410 ± 30*	9.4 ± 0.4*†
7D	15.7 ± 0.2*†	92.3 ± 2.8†	107.3 ± 6.5†	76.6 ± 1.6*†	156.8 ± 0.9*†	596 ± 28*	11.0 ± 0.5*†
28D	15.8 ± 0.3*†	130.9 ± 7.5*†	112.6 ± 3.7†	80.0 ± 1.3*†	319.5 ± 0.9*†	1,452 ± 67†	19.3 ± 1.0†

Values are means ± SE (6 animals in all groups; except 2H with 5 animals). Left and right soleus (Sol) muscles were paired in all the data collection. Ambulatory control (CTR), 0H, 2H, 1D, 3D, 7D, and 28D represented the time of reloading in hours (H) or days (D) in each group. *Significantly different from CTR. †Significantly different from animals 0H ($P < 0.05$). $RT_{1/2}$, one-half relaxation time; TPT, time to peak tension; P_t , twitch tension; P_o , tetanic tension; L_f , fiber length.

(Fig. 1, B and D, and Fig. 2, A and B). The concentration of neutrophils at 3 days was significantly lower relative to 1 day, whereas ED1⁺ concentration was not significantly different at 3 days relative to 1 day. The proportion of muscle fibers that were invaded by neutrophils and/or ED1⁺ macrophages also peaked after 1 day of reloading (Fig. 1, B and D, and Fig. 2D), but it never exceeded 0.1 and 0.5% of the total number of fibers, respectively. ED2⁺ macrophage concentration increased progressively to reach a significant difference at 3 days of reloading (Figs. 1F and 2C).

Correlation between the time courses of maximum P_o and inflammatory cell accumulation. The values for normalized P_o (N/cm²) were significantly altered by 10 days of HS as a 30% decrease was observed compared

with ambulatory control (Fig. 3). Although degradation and changes in structural and contractile proteins are probably responsible for the progressive decline in P_o during the 10-day unloading period, they are unlikely to be the main factors explaining the additional 50% loss in P_o observed after only 2 h of reloading. The loss in P_o at 2 h of reloading was not temporally associated with a significant elevation in muscle inflammatory cell concentrations. This lack of temporal relationship may indicate that inflammatory cells do not contribute to muscle impairment during early reloading (Fig. 3).

E-C coupling failure during early reloading period. The purpose of the caffeine experiments was to determine if failure or impairment of the E-C coupling could be involved in the P_o decrement observed in Sol mus-

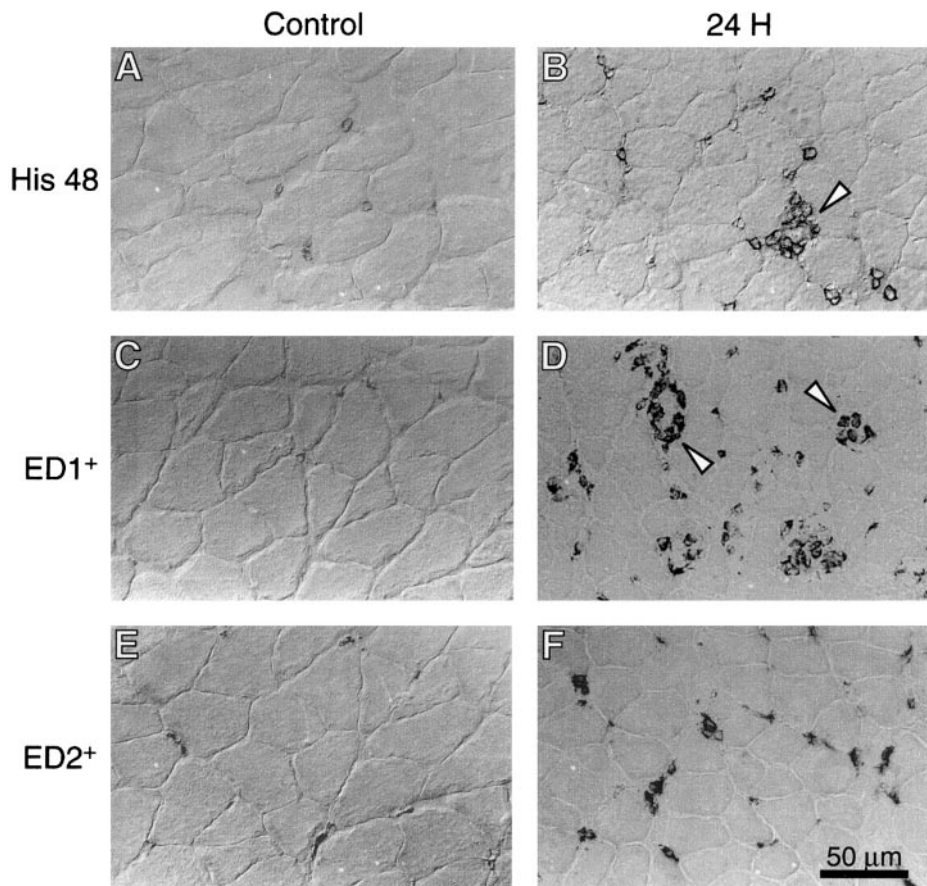
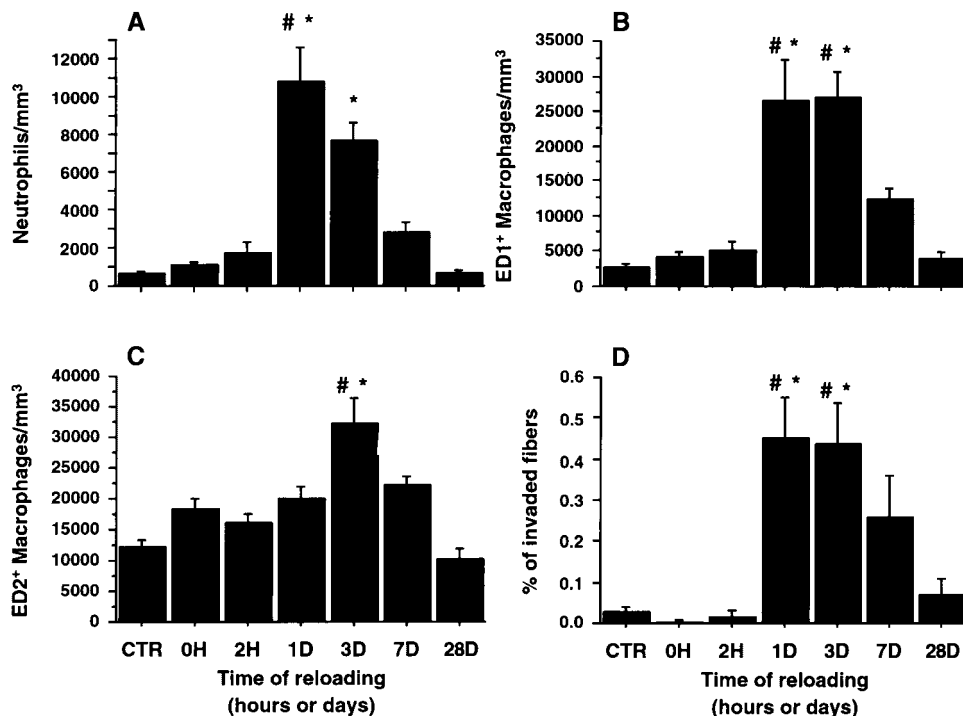


Fig. 1. Immunolabeling of neutrophils, ED1⁺, and ED2⁺ macrophages. Left: cross sections of soleus muscles from ambulatory control animals labeled with anti-His-48 (A), anti-ED1⁺ (C), or anti-ED2⁺ (E). Right: soleus muscle after 24 h of reloading, labeled with anti-His-48 (B), anti-ED1⁺ (D), or anti-ED2⁺ (F). Arrowheads indicate muscle fiber invasions by neutrophils and ED1⁺ macrophages.

Fig. 2. Inflammatory cell concentrations and muscle fiber injury following modified loading. A: neutrophil concentration in soleus muscle. B: ED1⁺ macrophage concentration in soleus muscle. C: ED2⁺ macrophage concentration in soleus muscle. D: muscle fiber necrosis expressed as percentage of total fibers in soleus muscle. Fibers invaded by ED1⁺ macrophages were counted as necrotic. CTR, ambulatory control; 0H, animal subjected to 10 days of hindlimb unloading. 2H, 1D, 3D, 7D, and 28D groups represent the period of reloading in hours (H) or days (D) following 10 days of hindlimb suspension. *Significantly different from CTR. #Significantly different from suspended animals. Data are means ± SE.

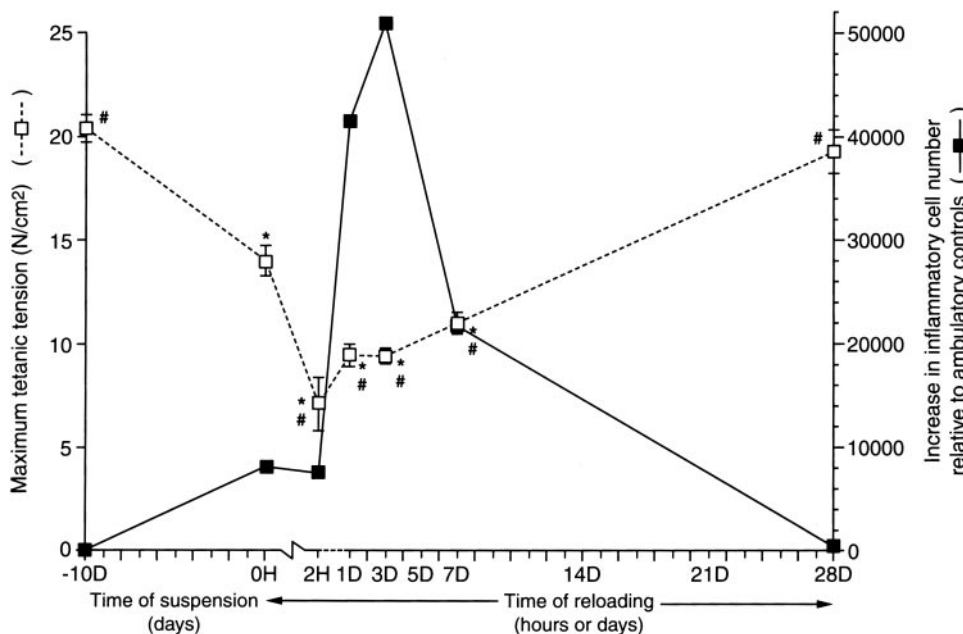


cles during the early reloading period. Caffeine acts directly at the level of SR to cause Ca²⁺ release independently of several steps of the E-C coupling cascade. We performed caffeine experiments on Sol muscles of ambulatory control animals. In accordance with a previous observation, the ratio of caffeine/P₀ force obtained in Sol muscles from ambulatory control animals is 0.54. The important discrepancy between control and experimental muscles in our model is very interesting and possibly related to changes in phenotype expression toward fast-twitch fibers during the period of suspension. Fast-twitch muscles are two to three

times less responsive to caffeine than slow-twitch muscles (14). Current evidence has recently demonstrated that different responses to caffeine may be associated with the presence of ryanodine receptor-3 (RyR3) in slow-twitch fibers (25). Because of the profound change from slow- to fast-twitch fibers during the period of suspension, we selected the unloaded animals as controls for the evaluation of the effect of reloading on muscle function.

In this set of experiments, the absolute P₀ decreased significantly by more than 40% between 0 and 2 h of reloading, whereas caffeine-induced force did not

Fig. 3. Time course of maximum tetanic tension and inflammatory cell accumulation in soleus muscle. Maximum tetanic tension was obtained at 25°C in soleus muscles from ambulatory controls and in animals submitted to modified muscle use. After measurements of contractile properties, soleus muscle was frozen, sectioned, and labeled for neutrophils, ED1⁺, and ED2⁺ macrophages. Total increase in inflammatory cell number is represented and expressed relative to ambulatory control. *Significantly different from CTR. #Significantly different from suspended animals. Data are means ± SE.



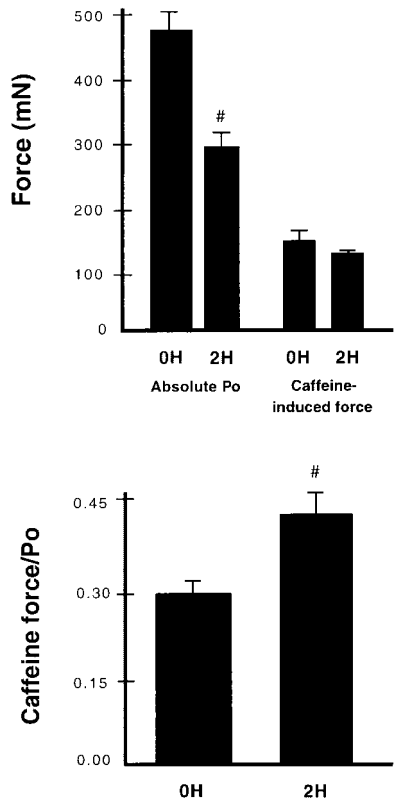


Fig. 4. *Top*: maximal isometric tetanic force (P_o) and caffeine-induced force in soleus muscles following hindlimb suspension and reloading. Caffeine (50 mM) was added to a Krebs-Ringer bicarbonate buffer, and passive tension was recorded every 2 min until maximum tension was produced. 0H, hindlimb suspension for 10 days; 2H, hindlimb suspension for 10 days followed by 2 h of reloading. *Bottom*: ratio of caffeine-induced force to P_o following modified mechanical loading. #Significantly different from suspended animal. Data are means \pm SE.

change significantly over time (Fig. 4, *top*). The ratio of caffeine-induced force/ P_o was 0.30 in muscle deprived of mechanical loading for 10 days, and it increased to 0.42 after only 2 h of reloading (Fig. 4, *bottom*). Because no significant changes in fiber type are possible during the first 2 h of reloading, we estimated that at least 40% of the P_o decrement is associated with a loss of efficiency of the E-C coupling process.

DISCUSSION

The importance of neutrophils in muscle injury has been confirmed in ischemia/reperfusion injuries (17, 29), but a similar implication of leukocyte subsets has not been convincingly demonstrated during modified muscle use (33). In a recent investigation, it was shown that ED1⁺ macrophage invasion does not contribute to muscle membrane injury during inflammation (33). Here we show that neutrophil and macrophage accumulation did not coincide with the rapid force decrement seen 2 h after reloading. This observation supports the hypothesis that the initial mechanism of reloading-induced injury is a mechanically mediated event in which inflammatory cells play a minor role (20). Although inflammatory cell concentration and

muscle function were not repeatedly evaluated to coincide with the peak of inflammatory cell accumulation for each subset of leukocytes, it was unexpected to observe that the total inflammatory cell accumulation was not associated with a second loss in muscle force. A causal relationship between muscle impairment and inflammatory cell accumulation remains possible for other unselected time points, but the recuperation of muscle force production beyond 2 h of reloading suggests that the inflammatory process is well controlled and that limited amounts of potentially harmful materials are released by leukocytes. The resolution of the inflammatory response without obvious damage or significant scarring formation has been found in lungs after the instillation of *Streptococcus pneumoniae* organisms (10), indicating that leukocytes can be eliminated by phagocytosis before they release a variety of destructive agents such as reactive oxygen species (19) and proteases (11). Macrophages have been shown to phagocytose intact apoptotic and senescent neutrophils (26), and there is evidence that a similar controlling mechanism of cell death occurs in reloaded Sol muscles thereby preventing tissue from a secondary loss in force associated with neutrophil activation (34).

The E-C coupling process can be fractionated into several distinct steps that include: 1) propagation of the sarcolemmal action potential, 2) t-tubular charge movement, 3) coupling of t-tubular charge movement with Ca^{2+} release from the SR owing to dihydropyridine receptor (DHPR) present in t-tubular membrane and RyR of the SR, and 4) Ca^{2+} release from the SR. With the use of a protocol of eccentric exercise-induced muscle damage, others have demonstrated that impairment of sarcolemmal function was not implicated in the E-C coupling failure induced by such a protocol (*step 1*) (36). These same investigators also showed that access to the t-tubular membrane and lumen was preserved, and only transient damage deep within the t-tubular system was observed following such an injury protocol (*step 2*) (37). Although the exact site of the defect in the E-C coupling process has not yet been experimentally determined in the exercise-induced muscle damage, the site of E-C coupling failure probably lies between the t-tubular voltage sensor and the SR Ca^{2+} release channel (*step 3*). This assumption is based on the observations that P_o and potassium-induced muscle contracture by depolarizing t-tubular membranes adjacent to the voltage sensors were proportionally reduced following eccentric contractions (13). Because the Sol muscle is significantly shorter in length following 10 days of HS, this would inevitably produce sustained eccentric contractions on reloading. Repeated stretching of a short Sol during reloading could result in muscle damage and E-C coupling failure. The caffeine experiment reveals that at least 40% of the P_o decrement between 0 and 2 h of reloading originates from a failure in the E-C coupling process at a step preceding the opening of the SR Ca^{2+} release channel. Moreover, the contribution of the E-C coupling process to the loss of muscle function is probably

underestimated in our experiment, because caffeine cannot activate entirely all fibers simultaneously.

At first glance, our results seem to contradict Ingalls et al. (12) who showed that the reduction in muscle force after HS was not exacerbated by 24 h of reloading as it resulted in only a minor impairment of E-C coupling in mouse previously deprived of mechanical loading for 14 days. This apparent discrepancy may be related to the time point selected in that study, which was probably too late to allow them to note the early specific force loss associated with E-C coupling failure during the reloading period. Another factor that could explain part of the discrepancy is the fact that there is a very important difference in muscle fiber type composition between Sol muscles from a mouse and rat. Sol muscle is composed of 85% type I fibers in the rat (2), whereas only 35% of the fibers are of type I in the mouse with the remaining being type IIa fibers (9). Because slow oxidative fibers are significantly more affected by HS (5) and preferentially damaged during the reloading period (35), it may therefore not be surprising to observe a more important impairment in Sol muscles from the rat compared with mice.

In agreement with others (5, 8, 27), TPT and $RT_{1/2}$, two contractile property measurements influenced by how Ca^{2+} is handled by the SR, were 25 and 38% faster, respectively, following HS in the present study. One mechanism by which speed of contraction may be affected is by influencing the expression of several key E-C coupling proteins during the absence of mechanical stress. For example, the level of α_1 -subunit of DHPR mRNA in Sol muscles was increased by 80% after only 24 h of HS without changes in muscle mass (15). HS for a duration of 14 or 28 days stimulated the expression of DHPR mRNA by 200% in Sol muscles to a level indistinguishable from those normally observed in extensor digitorum longus muscles, a muscle primarily composed of fast-twitch fibers. The significance of changes in the speed of contraction is also evidenced by the upregulation of the mRNA and protein levels of the fast SR Ca^{2+} pump following HS (27).

In summary, we conclude in this model of HS followed by reloading that 1) the increase in inflammatory cell concentration is not associated with any drop of muscle force, 2) the inability to activate the contractile machinery is the primary mechanism for the loss in force production early after reloading, and 3) further investigation using more direct approaches is warranted to determine the exact site of the defect in the E-C coupling failure.

Perspectives

One objective of the United States and international space programs is to undertake a human explorative mission on Mars by the year 2014. To reach this goal, the deleterious effects of microgravity on bones, skeletal muscles, and other organs must be clarified. The results of the present study showed that the loss in muscle force occurs before the invasion of inflammatory cells, suggesting that drugs directed to alleviate

inflammation may not be necessary for astronauts returning to earth. The present findings may also be applicable to other clinical situations in which patients confined to a wheelchair, bed, or immobilized in a cast experience a period of unloading followed by a reloading period. Further studies are warranted to identify different treatments that can prevent this rapid loss in muscle function following modified mechanical loading.

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