Neutrophil-induced skeletal muscle damage: a calculated and controlled response following hindlimb unloading and reloading

Nicolas Dumont,1 Patrice Bouchard,1 and Jérôme Frenette1,2

1Centre Hospitalier Universitaire de Québec-Centre de Recherche du Centre Hospitalier de l’Université Laval, Université Laval, Quebec City, Quebec, Canada; and 2Département de Réadaptation, Faculté de Médecine, Université Laval, Quebec City, Quebec, Canada

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Dumont N, Bouchard P, Frenette J. Neutrophil-induced skeletal muscle damage: a calculated and controlled response following hindlimb unloading and reloading. Am J Physiol Regul Integr Comp Physiol 295: R1831–R1838, 2008. First published September 10, 2008; doi:10.1152/ajpregu.90318.2008.—Neutrophils phagocyte necrotic debris and release cytokines, enzymes, and oxidative factors. In the present study, we investigated the contribution of neutrophils to muscle injury, dysfunction, and recovery using an unloading and reloading model. Mice were submitted to 10 days of hindlimb unloading and were transiently depleted in neutrophils with anti-Ly6G/Ly6C antibody prior to reloading. Leukocyte accumulation and muscle function were assessed immunohistologically and functionally in vitro. In addition, soleus muscles submitted to unloading and reloading were incubated in vitro with LPS (100 μg/ml) to determine whether exogenous stimulus would activate neutrophil response and produce extensive muscle damage. Contractile properties were recorded every hour for 6 h, and muscles were subsequently incubated in procion orange to assess muscle damage. Neutrophil depletion affected neither the loss in muscle force nor the time of recovery in atrophied and reloaded soleus muscles. However, atrophied and reloaded soleus muscles that contained high concentration of neutrophils experienced a 20% greater loss in force than atrophied and reloaded soleus muscles. In contrast, the stimulation of infiltrated neutrophils with LPS induced a 2.5-fold increase in muscle membrane damage in the presence of LPS. These results show that neutrophil infiltration during modified mechanical loading is highly regulated and efficiently eliminated, with no significant muscle fiber injury unless the activation state of neutrophils is modified by the presence of LPS.

In skeletal muscle and possibly other tissues, leukocyte recruitment varies in number and type according to the insult. For example, ischemia and reperfusion of skeletal muscle provoke a massive recruitment of neutrophils with a mild invasion of monocytes/macrophages. Conversely, eccentric contractions are known to induce important muscle damage and macrophage infiltration, but the extravasation of neutrophils is still debated (34, 52). Other protocols like hindlimb unloading and reloading or chemical injury with bupivacaine leads to a classic infiltration of neutrophils followed by macrophages (19, 46). Furthermore, the contribution of different subsets of leukocytes to muscle injury and recovery has partly been unraveled following muscle crush and chemical and ischemic muscle injury (2, 33, 50, 52). However, the hindlimb unloading and reloading model has provided conflicting results relative to neutrophil infiltration and muscle injury. Previous studies have shown that hindlimb unloading followed by reloading causes very little muscle fiber damage (1–2%), despite the presence of high numbers of neutrophils (18, 19, 45, 61). In addition, in vitro functional and immunohistological analyses also indicate that the loss in muscle force associated with hindlimb unloading and reloading does not correlate with the well-documented time course of inflammatory cell accumulation (19). It is tempting to speculate that the complex network of regulatory signals, which coordinates inflammation, would differ according to the initial and ongoing cues. Indeed, the physiological processes regulating muscle atrophy and recovery in unloaded and reloaded muscles are certainly different from muscle crush and chemical and ischemic injuries, and they could consequently lead to a distinct neutrophil response. In the former situation, there is basically no muscle damage, and the goal is to maximize muscle recovery, whereas the latter cases require breakdown of muscle fibers, phagocytosis, satellite cell proliferation, myotube formation, and finally tissue remodeling to reestablish homeostasis.

The objectives of the present investigation were thus 1) to determine the effects of neutrophils on muscle dysfunction and recovery following unloading and reloading periods and 2) to examine whether changes in the microenvironment can escalate the neutrophil response in reloaded soleus muscles in vitro. Our results indicate that the presence of neutrophils did not affect muscle mass and function during the inflammatory and recovery phases following unloading and reloading periods. Conversely, the stimulation of infiltrated neutrophils with LPS in vitro induced muscle damage and loss in muscle force.

The basic defense functions of neutrophils include phagocytosis of cell debris and invading microorganisms, release of proteolytic enzymes, and generation of oxygen-derived reactive agents (40, 44). In response to specific stimuli, neutrophils also synthesize and release an array of soluble cytokines and other mediators (8, 17, 53, 57, 58) that orchestrate the onset and progressive phases of inflammation, in part, through the recruitment and/or activation of several cell types, including neutrophils themselves, mononuclear cells, platelets, endothelial cells, fibroblasts, and muscle cells (31). During the normal course of inflammation and wound healing, macrophages replace neutrophils and become the most numerous leukocytes in the exudates (23, 24).

Address for reprint requests and other correspondence: J. Frenette, Centre Hospitalier Universitaire de Québec-Centre de Recherche du Centre Hospitalier de l’Université Laval, 2705 Boulevard Laurier, T-R-93, Quebec City, QC, Canada G1V 4G2 (e-mail: jerome.frenette@crchul.ulaval.ca).

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Together, these results demonstrated that microbial products or mechanical loading can activate invading neutrophils to different degrees in skeletal muscle, thereby directly influencing the outcome of muscle recovery.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (22–24 g) from Charles River (St-Constant, Quebec, Canada) were housed with water and food ad libitum and maintained with a 12:12-h light-dark cycle. Experimental animals were subjected to hindlimb unloading for 10 days, using a modification of the technique developed by Morey-Holton and Globus (41). To ensure normal hydration, the unloaded mice received 0.4 ml of saline solution containing 5% dextrose intraperitoneally every day during the suspension period. The mice were then reloaded for 1, 3, or 14 days. Ambulatory controls were also used. Mice were euthanized by cervical dislocation under anesthesia. All the procedures were approved by the Université Laval Research Center Animal Care and Use Committee based on the guidelines of the Canadian Council on Animal Care.

Neutrophil depletion and flow cytometry analysis. To determine whether neutrophil depletion encompasses the entire period of neutrophil infiltration, test mice were injected with 50 μl (0.025 mg) of rat anti-mouse Ly6G/Ly6C (Gr-1) antibody, clone RB6-8C5, (BD Pharmingen, Franklin Lakes, NJ) 24 h before and after reloading (5). Previous findings showed that this Ly6G/Ly6C antibody depletes specifically mature granulocytes, especially neutrophils and does not influence total monocyte/macrophage count (10, 12, 26, 35, 48, 54). Placebo mice were injected with 50 μl of rat serum (Sigma, St. Louis, MO). Neutrophil depletion was confirmed by flow cytometry. In brief, 0.5 to 1 ml of blood was collected by cardiac puncture under anesthesia with a ketamine-xylazine cocktail and kept at room temperature in Microvette 500 tubes coated with EDTA (Sarstedt AG & Co., Nümbrecht, Germany). Erythrocytes were lysed in 100 volumes of hemolysis buffer containing 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, pH 7.3. The cells were then centrifuged at 500 g for 5 min at room temperature. The supernatant was discarded, and the pellet was suspended in 500 μl of cold PBS. The cells were again centrifuged at 500 g for 5 min at 4°C. The two previous steps were repeated, and the pellet was suspended in 1.5 ml of cold PBS containing 1% bovine serum albumin (13). To allow the detection of neutrophil, cells were incubated with 0.2 mg R-phycocerythrin-conjugated rat anti-mouse Ly6G monoclonal antibody (primary antibody, BD Pharmingen) and 0.2 mg R-phycocerythrin-conjugated rat anti-mouse IgG, k immunoglobulin (isotype control, BD Pharmingen) for 20 min. The cells were washed twice with blocking buffer; Serotec, Oxford, UK) to identify macrophages, or with rat anti-mouse Ly6G/Ly6C (diluted 1/300 in blocking buffer) to identify neutrophils. They were then labeled with rat anti-rat biotinylated IgG (diluted 1/200 in PBS; Vector Laboratories, Burlingame, CA) for 1 h, incubated with horseradish peroxidase avidin D (diluted 1/1,000, Vector Laboratories) for 30 min, and revealed with DAB Chromogen (DakoCytomation, Carpinteria, CA). Each section was examined at a magnification of ×400, and the total number of labeled cells was determined and expressed per millimeters squared. The number of inflammatory cells was measured in duplicate on two midbelly sections from both the left and right soleus muscles. The average of the four sections per mouse per antibody was thus used for statistical analysis.

Assessment of muscle fiber injury. Sections of midbelly and the proximal/distal ends of muscles incubated with procion orange were cut at 10 μm thick and were adhered to Snowcoat X-tra slides (Surgipath, Richmond, IL). Erythrocytes were lysed using a digital imaging system (ImageJ, National Institutes of Health, Bethesda, MD). Laminin antibody stains muscle fiber basement membrane, while the presence of orange dye inside muscle fibers indicates fiber damage and membrane disruption. Background staining was evaluated in control soleus muscles, and the average value was set as the threshold for determining the number of injured fibers. Muscle fibers with a staining intensity above the threshold were

Table 1. Depletion of circulating neutrophils following Ly6G/Ly6C antibody injection

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Granulocytes, %</th>
<th>Labeled Cells, %</th>
<th>Number of Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.43</td>
<td>64.9</td>
<td>806</td>
</tr>
<tr>
<td>Once depleted</td>
<td>9.33</td>
<td>60.9</td>
<td>463</td>
</tr>
<tr>
<td>Twice depleted</td>
<td>4.53</td>
<td>17.5</td>
<td>42</td>
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This experiment included three groups with 3 independent experiments in each group: control mice injected with rat serum, mice injected with neutrophil antibody at day 1, and mice injected with neutrophil antibody at days 1 and 3. Blood samples were collected by cardiac puncture on day 3, which encompassed the period of neutrophil infiltration in muscle tissues. Number of cells in the granulocyte area/number of cells counted × 100. 

The number of labeled neutrophils in the granulocyte area.
classified as injured. The results were expressed as the percentage of injured fibers in the entire muscle section.

**Myeloperoxidase assays.** After 4 h of incubation with or without LPS, muscles were embedded in aluminum foil, frozen in liquid nitrogen, and kept at −80°C until the myeloperoxidase assay (maximum 48 h after the incubation). According to Lapointe et al. (33, 34), tissues were first weighed and homogenized in 10 volumes of a phosphate buffer containing: 40 mM K2HPO4, 60 mM KH2PO4, 0.1% Triton X-100, and 0.5% hexadecyltrimethylammonium bromide, pH = 7 (34). Muscles were then centrifuged at 850 g for 12 min (4°C). After the centrifugation, supernatants were twice frozen in liquid nitrogen for 2 min and thawed in warm water (37°C) for 3 min. Samples were centrifuged one more time, and 20 µl of supernatant was added to 180 µl of a reaction mixture containing 10 mM KH2PO4, 0.4 mM 0-dianisine dihydrochloride, and 0.8 mM of 30% hydrogen peroxide solution, pH = 6 for spectrophotometric analysis (Beckman DU-640). The absorbance was read at 480 nm at 37°C, and measurements were taken every 30 s for 180 s. One unit of enzymatic activity (U) corresponds to 1 mmol of substrate transformed to product per minute.

**Statistical analysis.** All values are reported as means ± SE. The data were analyzed by one-way ANOVA to determine whether the variations among the experimental groups were significant. When a significant F ratio was obtained, a post hoc multiple comparison was performed using Fisher’s protected least-significant differences test to determine whether specific differences had occurred. The level of significance was set at *P* < 0.05 (Statview, SAS Institute, Cary, NC).

**RESULTS**

Our results showed that 2 Ly6G/Ly6C antibody injections were required to maintain constant depletion over 5 days. The neutrophil population of mice that had received two injections of the antibody was depleted by ∼95% at 5 days posttreatment (Table 1). At this time point, the quantification of circulating neutrophils by flow cytometry revealed high numbers of circulating neutrophils, exhibiting strong fluorescence in control mice injected with PBS and mice injected once with antineutrophil antibody (Fig. 1, A and B). The second peak, which corresponds to the number of neutrophils in the granulocyte area, was almost nonexistent in mice injected twice with the antibody, indicating that the neutrophils had been almost completely depleted (Fig. 1C).

As reported previously (14, 19), unloading and reloading induced a sequential infiltration of neutrophils and macrophages. The number of neutrophils was roughly 300% higher in the experimental mice 1 day postreloading than in the ambulatory control mice (Fig. 2A) and remained significantly higher 3 days postreloading. The number of macrophages was 600% and 700% higher in the experimental mice 1 and 3 days postreloading, respectively, than in the ambulatory controls (Fig. 2B). The concentration of both neutrophils and macrophages returned to control values 14 days postreloading. In control mice, the basal number of neutrophils in soleus muscles was reduced by 80% following Ly6G/Ly6C injections, relative to undepleted mice. However, the density of macrophages in soleus muscles of control mice was not affected by the Ly6G/Ly6C injections. In addition, the kinetics and magnitude of neutrophil trafficking in response to unloading and reloading were almost completely abrogated in mice injected with the antineutrophil antibody. The depletion of neutrophils also reduced the concentration of macrophages by 35% in soleus muscles reloaded for 1 day compared with the undepleted group (Fig. 2B). The number of macrophages in mice depleted in neutrophils reached the undepleted value after 3 days of reloading.

Ten days of unloading followed by 1 day of reloading caused a 50% decrease in muscle mass and muscle force, which was similar to previous results obtained with rat soleus muscles (14, 19). These morphological and physiological changes were still present after 3 days of reloading but returned to control values after 14 days. The presence of neutrophils had

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**Table 2. Absolute muscle force at initial time (0 h) of soleus muscles incubated 6 h with or without LPS**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Absolute Force, g</th>
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<tr>
<td>Control and neutrophil-undepleted soleus without LPS</td>
<td>19.5±1.4</td>
</tr>
<tr>
<td>Control and neutrophil-undepleted soleus with LPS</td>
<td>15.7±2.5</td>
</tr>
<tr>
<td>Reloaded and neutrophil-undepleted soleus with LPS</td>
<td>6.5±1.2*</td>
</tr>
<tr>
<td>Reloaded and neutrophil-depleted soleus with LPS</td>
<td>7.9±0.6*</td>
</tr>
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Values are expressed as means ± SE (n = 5). *Significantly different from both control neutrophil-undepleted soleus incubated or not with LPS, P < 0.05.

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**Fig. 1. Charts representing the quantification of circulating neutrophils by flow cytometry with R-phycoerythrin-conjugated rat anti-mouse Ly6G monoclonal antibody 5 days posttreatment.**

A: neutrophils labeled with fluorescent dye exhibited strong fluorescence and high cell counts in control mice injected with PBS (see peak A). B: similar result was observed with mice injected once with Ly6G/Ly6C antibody (see peak G). C: second peak (K) was almost nonexistent for mice injected twice with the antineutrophil antibody, indicating significant neutrophil depletion. Peaks B, H, and L represent nonspecific fluorescence.
no impact on muscle dysfunction and recovery, as no significant differences in muscle force and muscle mass were observed between neutrophil-depleted and -undepleted mice (Fig. 3, A and B).

To determine whether neutrophil activation was submaximal in our model of modified mechanical loading and whether secondary muscle damage could be initiated with exogenous stimulus, soleus muscles were incubated in oxygenated Krebs-Ringer solution with or without LPS, and muscle force was recorded for 6 h. This period of incubation was selected because it allows enough time for LPS penetration and neutrophil activation, with limited effect on muscle viability.

Indeed, muscle force production from neutrophil-undepleted control soleus muscles (11.3 neutrophils/mm²) was still close to 70% of its initial maximal force after 6 h of incubation in oxygenated solution without LPS at 37°C (Fig. 4). However, the addition of LPS increased the loss of muscle force to 50% after 6 h of incubation. Similar and significant differences in muscle force production were also observed between unloaded and reloaded soleus muscles depleted in neutrophils (2.4 neutrophils/mm²) and unloaded and reloaded soleus muscles undepleted in neutrophils (29 neutrophils/mm²) incubated with LPS. Indeed, soleus muscles that contained high neutrophil density showed a 50% decrease in muscle force after 6 h relative to a 30% decrease in soleus muscles depleted in neutrophils (Table 2). The significant differences in force production between neutrophil-undepleted and -depleted soleus muscles appeared after a 4-h incubation in LPS. To further validate the effect of neutrophils on muscle damage, soleus muscles were stained with procion orange (Fig. 5; see light gray areas). These results corroborated the data on contractile properties and showed a much higher percentage of injured muscle fibres in LPS-incubated muscles compared to control muscles.
fibers in control soleus muscles incubated with LPS than control soleus muscles incubated without LPS (40% and 21%, respectively). The same striking difference could also be observed in reloaded soleus muscles stimulated with LPS where neutrophil-undepleted muscles had significantly more fiber injury than neutrophil-depleted ones (42% and 17%, respectively) (Fig. 6).

To confirm that the higher level of injury following muscle incubation in LPS is specifically related to neutrophil activation, myeloperoxidase assay (MPO) was performed (Fig. 7). The results demonstrate that soleus muscles from neutrophil-undepleted mice that were unloaded and reloaded following LPS stimulation. Furthermore, the MPO activity also increased by six-fold in neutrophil-undepleted mice incubated with LPS compared to those incubated without LPS. Data are expressed as means ± SE (n = 4). *Significantly different from control and neutrophil-undepleted mice with LPS, P < 0.05. **Significantly different from control and neutrophil-undepleted mice without LPS, P < 0.05. **Significantly different from reloaded and neutrophil-undepleted mice with LPS, P < 0.05.
shown that neutrophils are able to release MIP-1α, which influence macrophage recruitment (65). While neutrophils are important for macrophage recruitment and/or proliferation, the partial reduction of macrophage numbers in neutrophil-depleted mice indicates that other cell types contribute to macrophage infiltration and proliferation in soleus muscles. Injured muscle fibers, satellite cells, endothelial cells, endogenous macrophages, and mast cells are probably the best candidates (3, 9, 22, 43, 47). A previous study investigating mast cell activators and inhibitors showed that these cells participate, directly and/or indirectly, in neutrophil and macrophage recruitment in unloaded and reloaded soleus muscles (14). The presence of macrophages in sufficient numbers is particularly important for removing necrotic and apoptotic cells, for regulating inflammatory processes, and for eliciting myogenic precursor cell responses (2). Macrophages can specifically stimulate myoblast proliferation and survival by releasing mitogenic factors and antiapoptotic signals (9, 60). In addition, the depletion of circulating monocytes prevents muscle regeneration, whereas the selective and local depletion of intramuscular macrophages at later stages of healing reduces the diameter of regenerating fibers following chemical muscle injury (2). Because neutrophil depletion combined with a reduction in the number of macrophages did not have an impact on muscle injury and recovery following reloading, we can conclude that sufficient macrophages remained to perform their function in our model of muscle atrophy and recovery.

Recent results from our laboratory and others have shown that leukocyte recruitment in skeletal muscle varies in number and type, depending on the insult (19, 28, 34, 46, 51, 52). However, there is no correlation between the number of neutrophils and the extent of collateral muscle damage. Contrary to ischemic and reperfused skeletal muscles, neutrophil activation is highly regulated, and successful clearance of neutrophils occurs with limited muscle fiber damage during the reloading of atrophied soleus muscles (39, 62). One explanation for this lack of correlation is that skeletal muscle or other tissues may be able to induce distinct inflammatory responses in function with the type of insults (4). For example, an in vitro study, in which myotubes were either traumatized or strained, showed that both types of injury can induce the release of factors that influence neutrophil chemotaxis and priming but that only traumatized myotubes can release factors that activate neutrophils to produce free radicals (63). This indicates that proinflammatory molecules released by muscle cells, which vary depending on the type and intensity of injury, can activate neutrophils to different degrees (15). In addition, chemotactic substances released at sites of injury activate and upregulate cell surface adhesion molecules that support rolling, firm adhesion, and transmigration of leukocytes. Interestingly, the release of IL-8 and leukotriene B4 is associated with CD11a/CD18 integrin-dependent neutrophil migration, while the bacterial peptide fMLP and the complement C5a stimulate CD11b/CD18-dependent neutrophil migration (25, 42, 64). Regulating the expression of these adhesion molecules thus plays a major role in the localization, development, and intensity of inflammatory responses (36, 59). Indeed, blocking CD11b/CD18 integrin reduces not only neutrophil infiltration but also decreases the release of reactive oxygen species by infiltrated neutrophils following liver ischemia by more than 50% (29). Because modified loading periods provoke significant neutrophil infiltration with very minor muscle fiber leakage and necrosis (1 to 2%) (19, 61, 62), chemotactic factors released by muscle and nonmuscle cells may attract neutrophils without activating them enough to release significant concentrations of free radicals and proteases to induce muscle injury.

In the second section of this experiment, control and reloaded atrophied muscles were incubated 6 h with LPS to determine whether changes in the microenvironment can prime and activate neutrophils to induce skeletal muscle damage. The...
prolonged incubation of control soleus muscles without LPS induced a moderate decrease of muscle viability after 6 h, probably because of muscle hypoxia in the deepest region, as shown by procion orange staining (see light gray areas in Fig. 5). However, the measurement of MPO activity clearly indicates that the addition of LPS is able to specifically overstimulate neutrophils and that this activation is associated with a more important loss in muscle force and fiber damage in vitro. Indeed, the incubation of control soleus muscles without LPS provoked a 30% decrease in muscle force, which was combined histologically with a 20% muscle fiber damage. The presence of LPS almost doubled the number of muscle fiber damage and loss of muscle force. Together, these results suggest that there is a switch from specific and self-limiting action of neutrophils toward a destruction response in the presence of LPS. It is known that LPS can interact directly with action of neutrophils toward a destruction response in the damage and loss of muscle force. Together, these results shown by procion orange staining (see light gray areas in Fig. 5). However, the measurement of MPO activity clearly indicates that the addition of LPS is able to specifically overstimulate neutrophils and that this activation is associated with a more important loss in muscle force and fiber damage in vitro.

Indeed, the incubation of control soleus muscles without LPS induced a moderate decrease of muscle viability after 6 h, while limiting the damaging aspects of inflammation as much as possible (4, 44).

**REFERENCES**


