Mast cells can modulate leukocyte accumulation and skeletal muscle function following hindlimb unloading

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Dumont N, Lepage K, Côté CH, Frenette J. Mast cells can modulate leukocyte accumulation and skeletal muscle function following hindlimb unloading. J Appl Physiol 103: 97–104, 2007. Rodent hindlimb suspension is widely used to induce inflammation and muscle impairment. We set out to define the role of mast cells in neutrophil and macrophage recruitment and muscle recovery after unloading-reloading. We hypothesized that mechanical perturbation would stimulate release of proinflammatory substances by mast cells, which would influence leukocyte recruitment and muscle function. Rats were suspended for 10 days and injected with a mast cell inhibitor (cromolyn) or stimulator (compound 48/80) or a placebo before reloading. Leukocyte accumulation and muscle function were assessed using immunohistological staining and measurements of contractile properties in vitro. Our results showed that mechanical loading activated mast cells, thereby influencing leukocyte recruitment in the early reloading periods. Indeed, the inhibition of mast cell degranulation significantly reduced the number of neutrophil cell profiles in reloaded soleus muscle, whereas mast cell activation provoked a significant increase in the number of neutrophil cell profiles in uninjured muscle. However, the inhibition of mast cell degranulation also led to a significant increase in the number of ED1+ macrophage cell profiles. These perturbations in the inflammatory response caused by mast cell inhibition induced a short protective effect on the loss of muscle force after 1 day of reloading but delayed the return to the normal contractile properties of muscles after 14 days of reloading. These results indicate that mechanical loading can induce mast cell degranulation, which can influence leukocyte influx and muscle function, and also highlighted the possibility that leukocytes may play a dual role in skeletal muscles.

Mast cells are an important source of TNF-α and the injection of this cytokine provoked the recruitment of neutrophils and macrophages into soleus muscles of mice, it is becoming apparent that mast cells can play key roles in activating, recruiting, and directing leukocytes to skeletal muscles (30, 34, 35). We set out to define the role of mast cells in a well-described and well-defined model of HU in which inflammation and muscle recovery occur. We postulated that the mechanical stress associated with unloading-reloading stimulates endogenous mast cell degranulation and influences leukocyte recruitment and muscle function. We showed that the inhibition of mast cell degranulation decreased neutrophil accumulation in injured muscles, whereas mast cell activation induced neutrophil recruitment into uninjured soleus muscles. Lastly, a mast cell inhibitor provided a short-term protective effect on the loss of muscle after 1 day of reloading but delayed the return to normal contractile properties after 14 days of reloading.

MATERIALS AND METHODS

Experimental protocol. Female Wistar rats weighing 175–250 g at death were used for this study. The treatment and care of the animals were approved by and followed the guidelines of the University Hospital Research Center Animal Care and Use Committee. Rats were subjected to HU for 10 days, as described by Morey-Holton and

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Globus (31), followed by 1, 3, or 14 days of reloading. The rats were able to move with their forelimbs and had access to food and water ad libitum. They were suspended and randomly divided into three reloading groups: 1) cromolyn, a mast cell inhibitor, was injected in sterile PBS (160 mg/kg ip; Sigma, St. Louis, MO) 1 h before reloading and every 24 h for the first 3 days in the 3- and 14-day reloading groups, 2) compound 48/80 (CMP 48/80), a mast cell activator, was injected in sterile PBS (75 μg/100 g ip; Sigma) 3 h before reloading and every 24 h for the first 3 days in the 3- and 14-day reloading groups, and 3) sterile PBS was injected intraperitoneally (placebo). Control groups consisted of ambulatory rats injected intraperitoneally with cromolyn, CMP 48/80, or PBS (see above). Although the exact mechanism of mast cell stabilization by cromolyn is not fully understood, evidence suggests that it indirectly inhibits Ca$^{2+}$ from entering mast cells and triggering the release of cellular contents (21, 38). CMP 48/80 is a mixture of different-sized polymers derived from N-methyl-p-methoxyphenylamine. These compounds enter the lipid bilayer of the plasma membrane and directly activate G proteins linked to a phospholipase C signaling pathway, leading to efficient activation of mast cells (32).

Measurement of mechanical properties. At the end of the experimental protocol, the rats were injected with buprenorphine (0.1 mg/kg ip) ≥15 min before the administration of pentobarbital sodium (50 mg/kg). The right soleus was carefully removed, incubated in vitro in Krebs-Ringer bicarbonate buffer supplemented with 2 mg/ml of glucose, and incubated at 25°C with constant bubbling of carbogen (95% O$_2$–5% CO$_2$) to ensure viability. Isometric contractile properties were measured as previously described (9, 12, 36).

Tissue preparation and immunohistochemical and histological staining. After the measurement of mechanical properties, the left soleus was also removed and both soleus muscles were stretched near resting length, embedded in tissue-freezing medium, and frozen in isopentane cooled in liquid nitrogen. Sections were cut (10 μm) using a cryostat and labeled as described previously (15) with mouse anti-rat ED1 (CD68) and anti-ED2 (CD163) diluted 1:100 (Serotec, Raleigh, NC) and with mouse anti-rat W3/13 (CD43) diluted 1:50 (Serotec). Anti-ED1 and anti-ED2 recognize different subpopulations of macrophages (10), whereas W3/13 primarily recognizes neutrophils as well as other granulocytes (37). Thus the term neutrophil was used, because they are by far the most abundant leukocytes recognized by the antibody W3/13 in skeletal muscle. The density of labeled cells was quantified in two midbelly sections separated by 50 μm for each soleus muscle. The number of cell profiles was determined using a Nikon inverted microscope at ×400 magnification and was expressed as the number of cell profiles per square millimeter. A numbered grid divided into 100 squares was used to allow precise counting of inflammatory cell profiles and to avoid double counting. The area of this grid is 0.0625 mm$^2$ at ×400 magnification. For simplification of the assessment of cell profile density, muscle cross sections were divided into four equal areas from top to bottom. The first row of each section was systematically counted. Thus inflammatory cell populations were counted in the bottom, middle, and top portions of each muscle section. This method was used to count several hundred macrophage cell profiles (ED1$^+$ and ED2$^+$) and 20–100 mast cell and neutrophil cell profiles in each muscle section. For identification of mast cells, muscle sections were stained for 1 min in a toluidine blue solution: 2.5 ml of 65.4 mM toluidine blue (diluted in 70% ethanol) in 47.5 ml of 8.56 mM NaCl (diluted in distilled water). The tissues were washed three times in double-distilled water, dehydrated in xylene, and mounted in Eukitt mounting medium. Mast cells were considered degranulated when their granules were clearly visible outside the mast cell border (Fig. 1A). The number of degranulated mast cells was divided by the total number of mast cells in the section and expressed as a percentage.

Statistical analysis. The values of both soleus muscles were combined and reported as means ± SE. All 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 difference test to determine whether specific differences had occurred. The level of significance was set at $P < 0.05$.

RESULTS

Effect of HU and reloading on morphological and contractile properties. HU for 10 days followed by reloading for 1 day induced a ~50% decrease in muscle mass compared with the ambulatory group (Table 1). Muscle mass was still significantly decreased after 3 days but returned to control levels after 14 days. In addition, the time to peak tension (TPT, ms) and half-relaxation time (½RT, ms) diminished by 24 and 34%, respectively, after 10 days of HU followed by 1 day of reloading. More importantly, these periods of unloading and reloading provoked a 68% loss in absolute tetanic tension ($P_{0\text{t}}$, g).

Influence of mast cell inhibition on leukocyte accumulation and muscle function. We used two different experimental approaches to test the hypothesis that reloading-induced mast cell activation is involved in leukocyte recruitment: one consisted of inhibiting mast cell activation, and the other was designed to provide maximal mast cell stimulation. Consistent with our hypothesis, the change in mechanical stress and/or damage associated with reloading after HU did lead to mast cell activation. The percentage of degranulated mast cells of suspended and reloaded rats treated with cromolyn was much lower and very similar to that of the ambulatory controls at every time point (Fig. 1B). No difference in mast cell degranulation was observed between soleus muscles subjected to the contractile protocol and those subjected only to the histological protocol. As shown in Fig. 1C, the unloading-reloading protocol not only influences mast cell degranulation but, also, mast cell density. Indeed, the suspended groups showed a 1.5- to 2-fold increase in mast cell density 1 day after reloading compared with the ambulatory placebo group. A close analysis reveals that this increase in mast cell density is mainly caused by the reduction of muscle fiber size and total cross-sectional area. Furthermore, the mast cell inhibitor negatively influenced leukocyte accumulation during the reloading periods. The density of neutrophil cell profiles was reduced by 25% ($P < 0.05$) in cromolyn-treated animals compared with placebo-treated animals after 1 day of reloading (Fig. 2A). Conversely, accumulation of ED1$^+$ macrophage cell profiles was ~45% greater in muscles treated with the mast cell inhibitor than in those treated with placebo after 1 day of reloading (Fig. 2B). These opposite effects of cromolyn were limited 1 day after reloading. Lastly, no difference in the concentration of ED2$^+$ macrophage cell profiles was observed between rats treated with cromolyn and those treated with placebo at any time point (data not shown).

Although significant changes were observed in the proportion of degranulated mast cells and the density of neutrophil
and ED1⁺ macrophage cell profiles, these perturbations had little impact on contractile function. However, as shown in Fig. 3A, cromolyn reduced the decline in maximal absolute force production in soleus muscles by 17% (P < 0.05) after 1 day of reloading. This protective effect was no longer apparent after 3 days. TPT and ½RT returned to control values after 14 days in the suspended placebo animals but remained 15 and 23%, respectively, below control values, in the cromolyn-treated animals (Fig. 3B).

**Impact of mast cell activation by CMP 48/80 on leukocyte accumulation and muscle function.** CMP 48/80 had a rapid and very significant positive effect on the proportion of degranulated mast cells in ambulatory rats. The maximum effect was obtained 60–90 min after injection, with ~60% of the mast cell population having a degranulated appearance (Fig. 4). On the other hand, CMP 48/80 caused no change in the number of neutrophil cell profiles in suspended animals compared with placebo animals 1 and 3.
days after reloading. Interestingly, CMP 48/80 provoked a significant increase in neutrophil cell profile concentration in ambulatory rats 1 and 3 days after reloading that was still significant at 14 days after injection (Fig. 5A). ED1\(^+\) and ED2\(^+\) macrophage cell profile concentrations were not affected by mast cell stimulation in any of the groups at any time point (Fig. 5B; data not shown for ED2\(^+\)). Furthermore, CMP 48/80 had no effect on contractile properties (data not shown).

**DISCUSSION**

Leukocyte recruitment to sites of injury is coordinated by a complex interplay between resident and endothelial cells that regulates the expression of specific chemokine and adhesion molecules (8, 39). By virtue of their capacity to release potent mediators, mast cells are seen as key components for activating, recruiting, and directing leukocytes to extravascular tissues. Recent findings indicate that mast cells are able to communicate and recruit neutrophils (42, 44). The strategic localization of mast cells in the proximity of blood vessels, combined with their capacity to secrete preformed and newly synthesized molecules (16), makes them an excellent candidate for modulating leukocyte recruitment and muscle function in the ground-based model of hypogravity.

**Mast cell and neutrophil recruitment.** The findings presented here clearly indicate that modified mechanical loading can activate mast cells and influence leukocyte recruitment. Indeed, the proportion of activated mast cells increased threefold in soleus muscles after 1 day of reloading, which coincides with maximal neutrophil recruitment. Interestingly, the unloading and reloading periods were sufficient to activate mast cell levels similar to those induced by CMP 48/80, a potent mast cell activator. Although the administration of CMP 48/80 to ambulatory control animals induced a significant increase in neutrophil cell profile density in the absence of muscle atrophy, damage, and regrowth, CMP 48/80 did not potentiate neutrophil accumulation in reloaded and atrophied soleus muscles. This observation suggests that mast cell activation was already maximal in reloaded muscles and CMP 48/80 had no additive effect. Although the factors that can provoke mast cell degranulation in this model are unclear, we believe that mechanical perturbation can directly stimulate mast cells anchored to the extracellular matrix. Indeed, our preliminary experiments in

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**Table 1. Impact of hindlimb unloading and reloading on muscle mass and contractile characteristics**

<table>
<thead>
<tr>
<th></th>
<th>10 Days Suspension</th>
<th>Ambulatory Control</th>
<th>1 Day Reloading</th>
<th>3 Days Reloading</th>
<th>14 Days Reloading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus mass, mg</td>
<td>92.3 ± 1.8</td>
<td>45.0 ± 1.8</td>
<td>60.7 ± 2.8(±)</td>
<td>97.2 ± 4.3(±)</td>
<td></td>
</tr>
<tr>
<td>TPT, ms</td>
<td>90 ± 3.8</td>
<td>68.2 ± 2.8</td>
<td>83.8 ± 3.5(†)</td>
<td>98 ± 3.1(‡)</td>
<td></td>
</tr>
<tr>
<td>1/2RT, ms</td>
<td>112.7 ± 6.4</td>
<td>73.8 ± 6.3(*)</td>
<td>100.2 ± 3.8(†)</td>
<td>123.8 ± 2.9(‡)</td>
<td></td>
</tr>
<tr>
<td>(P_\text{m}), g</td>
<td>119 ± 4.5</td>
<td>38.1 ± 2.8(*)</td>
<td>50.6 ± 2.6(*)</td>
<td>108.1 ± 5.7(†)</td>
<td></td>
</tr>
<tr>
<td>(P_\text{m}), N/cm(^2)</td>
<td>19.5 ± 0.6</td>
<td>11.8 ± 1(*)</td>
<td>12.5 ± 0.7(*)</td>
<td>15.1 ± 0.7(‡)</td>
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Values of both soleus muscles were combined and reported as means ± SE; \(n = 6\) in all groups; except for 3 days reloading, where \(n = 5\). TPT, time to peak tension; 1/2RT, half-relaxation time; \(P_\text{m}\), maximal tetanic tension. \(^*\)Significantly different from ambulatory control, \(P < 0.05\). \(^†\)Significantly different from 1 day reloading \(P < 0.05\). \(^‡\)Significantly different from 3 days reloading, \(P < 0.05\).

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**Fig. 2. A and B:** concentration of neutrophil and ED1\(^+\) macrophage cell profiles in soleus muscles after 10 days of hindlimb unloading followed by 1, 3, and 14 days of reloading. Rats were injected with mast cell inhibitor (cromolyn) or placebo. Ambulatory animals were used as controls. Values are means ± SE; \(n = 5–6\), except for suspended, reloaded (1 day), placebo and suspended, reloaded (1 day), cromolyn, where \(n = 10–11\) (A) and 7–9 (B). \(^*\)Significantly different from ambulatory placebo group, \(P < 0.05\). \(^#\)Significantly different from ambulatory placebo group, reloaded, placebo, \(P < 0.05\).
vitro indicate that cyclic mechanical loading can upregulate the expression of proinflammatory cytokines in mast cells, suggesting that mechanical stress is sufficient to initiate mast cell activation. However, some studies have also demonstrated that ischemia-reperfusion injury leads to the production of the complement factors C3a and C5a and that these factors are able to induce connective tissue mast cell activation (3, 11). It is then highly likely that mast cell activation in the unloading-reloading protocol might be caused by a combination of different stimuli.

On the other hand, the inhibition of mast cells with cromolyn decreased the number of neutrophil cell profiles in soleus muscles reloaded for 1 day by ~25%. The inhibitory effect on neutrophil recruitment is consistent with the notion that mast cells can rapidly release a wide variety of chemotactic and proinflammatory molecules after stimulation (42). For example, mast cells can release molecules such as histamine, TNF-α (46, 48), platelet-activating factor, and leukotriene B4 (16, 43), which initiate leukocyte rolling and firm adhesion through selectin and β2-integrin mechanisms (15, 47). Furthermore, a recent study also demonstrated that injection of macrophage inflammatory protein-2 into mouse cremaster muscles induces neutrophil recruitment, which is dependent on TNF-α released by mast cells (44). Neutrophil recruitment in reloaded soleus muscles was not totally abolished by mast cell inhibition, rather it was inhibited by 25%. We can postulate that mast cells

Fig. 3. A and B: maximal tetanic tension (P0) \( n = 4–6 \), except for suspended, reloaded (1 day), placebo and suspended, reloaded (1 day), cromolyn, where \( n = 10–11 \) and time to peak tension (TPT) and half-relaxation time \( (1/2 \text{RT}) \) in right soleus muscles \( n = 6 \) after 10 days of hindlimb unloading followed by 14 days of reloading. Rats were injected with mast cell inhibitor (cromolyn) or placebo. Ambulatory animals were used as controls. Values are means ± SE. *Significantly different from ambulatory placebo, \( P < 0.05 \). #Significantly different from suspended, reloaded, placebo, \( P < 0.05 \).

Fig. 4. Time course of mast cell degranulation after injection of CMP 48/80. Time 0 represents basal level in ambulatory control rats. Both soleus muscles were used. Values are means ± SE; \( n = 2 \), except for time 0, where \( n = 5 \).
may be critical for the first and early release of chemotactic signals after reloading because of their capacity to stock preformed proinflammatory molecules (TNF-α and histamine) (16). Moreover, resident macrophages, fibroblasts, muscle cells, and endothelial cells may also contribute to orchestrate the neutrophilic response (7, 16, 17, 23, 29, 30). The release of proinflammatory substances by activated mast cells can thus influence inflammatory cell migration directly by the release of various chemotactic factors or indirectly by the upregulation of adhesion molecules on endothelial cells.

Mast cells and macrophage accumulation. Surprisingly, cromolyn increased the number of ED1+ macrophage cell profiles in reloaded soleus muscles by ~45%. Cromolyn failed to inhibit ED1+ accumulation in macrophages, in sharp contrast to neutrophils. This disparity implies fundamental differences within leukocyte subsets. Indeed, the evaluation of cell type-specific adhesion molecules and cytokine responses for chemotaxis clearly highlights a striking difference between macrophages and neutrophils (19). For example, neutrophils are selectin dependent for rolling and use β2-integrin to transmigrate into injured tissues, whereas monocytes can transmigrate in the absence of P- and E-selectins and may rely on β1- and α4-integrins (4, 14, 28, 33). In addition, many pro- and anti-inflammatory substances can be released by activated mast cells and may have different or opposite effects on leukocyte locomotion (16, 22). To this extent, IL-8 can induce neutrophil chemorepulsion or chemoattraction, depending on its concentration at the site of injury (40). Another possibility is that this contradictory effect underlies a potential compensatory mechanism. Indeed, the reduction in phagocytic neutrophils in reloaded muscles may overstimulate the recruitment of macrophages to “catch up” for the phagocytosis of cellular debris. Clarification of the contradictory effect of mast cell inhibition on neutrophils and macrophages in skeletal muscle requires that all these possibilities be explored.

Modulation of inflammation and muscle function. In the present study, contractile properties were also measured to determine whether the modulation of leukocyte accumulation by a mast cell inhibitor affected the time course of muscle recovery. Mast cell inhibition significantly reduced the decrease in absolute Po1 day after reloading compared with the placebo group. However, this protective effect was no longer apparent 3 days after reloading. The smaller loss of muscle force 1 day after reloading was associated with decreases in mast cell activation and neutrophil accumulation. We speculate that this transient, short protective effect may be associated with excitation-contraction coupling (ECC) mechanisms. Indeed, previous results from our laboratory showed that part of the large drop in Po1 day after reloading is caused by a perturbation of ECC mechanisms during the early reloading periods (15). Furthermore, neutrophils and mast cells can release free radicals and proteolytic enzymes, which may perturb this mechanism (1). Indeed, hydrogen peroxide can stimulate Na+/Ca2+ exchange and deplete sarcoplasmic reticulum Ca2+ stores in isolated cardiac muscles, suggesting that free radicals can have a significant effect on cardiac ECC (20). Maximal force produc-

Fig. 5. A and B: concentration of neutrophil and ED1+ macrophage cell profiles in soleus muscles after 10 days of hindlimb unloading followed by 1, 3, and 14 days of reloading. Rats were injected with CMP 48/80 or placebo. Ambulatory animals were used as controls. Values are means ± SE (n = 6). *Significantly different from ambulatory placebo group, P < 0.05. ‡Significantly different from suspended, reloaded, placebo, P < 0.05. ‡Significantly different from ambulatory, CMP 48/80, P < 0.05.
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