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Downhill running in rats: influence on neutrophils, macrophages, and MyoD+ cells in skeletal muscle

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Abstract The accumulation of neutrophils and macrophages, as well as the activation of satellite cells, are early events following skeletal muscle injury. We examined the temporal relationship between changes in neutrophils, macrophages, and MyoD protein, a marker of satellite cell activation, after injurious exercise. Male rats ($n=47$) performed an intermittent downhill (–16% grade) running (17 m/min) protocol and the solei were obtained at 0, 2, 6, 24, 48, or 72 h post-exercise. Neutrophils, macrophages (ED1 and ED2), and MyoD+ cells were determined in muscle cross sections using immunohistochemistry. Downhill running increased ($P \leq 0.05$) the percentage of injured fibers and elevated blood creatine kinase activity. Neutrophils were elevated 18-fold relative to controls at 24 h post-exercise. ED1 macrophages were elevated four- and twofold at 24 and 48 h post-exercise, respectively. Neither ED2 macrophages nor MyoD+ cells were elevated post-exercise. These observations may indicate that elevations in neutrophils and ED1 macrophages after injurious exercise are not temporally associated with an increase in satellite cell activation.

Keywords Muscle inflammation · Muscle damage
Acute inflammation · Satellite cells · Muscle
regeneration

Introduction

Degenerative and regenerative processes are induced within skeletal muscle following overt injury. Muscle degeneration is characterized by cytoskeletal disrup-

tions, altered sarcolemmal permeability, and reduced muscle function (Frenette et al. 2002; Koh and Brooks 2001; Thompson et al. 1999). Muscle regeneration consists of activation, proliferation, and differentiation of satellite cells into myotubes, which ultimately fuse with existing myofibers or fuse together to form new myofibers (Hawke and Garry 2001; Seale and Rudnicki 2000).

The degenerative and regenerative events after overt muscle injury are accompanied by an increase in inflammatory cells (neutrophils and macrophages) in skeletal muscle. The majority of investigators that quantified the time course of change of neutrophils and/or macrophages in skeletal muscle used the hindlimb suspension-reloading model of muscle injury (Frenette et al. 2000, 2002; Tidball et al. 1999). Because hindlimb suspension causes muscle atrophy, dysfunction, and inflammation (Frenette et al. 2002; Kandarian et al. 1991; Tidball et al. 1999) prior to the injury induced by muscle reloading (Frenette et al. 2002; Thompson et al. 1999), the hindlimb suspension reloading model may not be an ideal paradigm for studying muscle injury and inflammation associated with exercise. Thus, more information is needed on the time course of change in both neutrophils and macrophages following injurious exercise before their biological function within skeletal muscle following exercise can be ascertained.

Although the biology of inflammatory cells within skeletal muscle is poorly understood, inflammatory cells are thought to contribute to the events associated with muscle regeneration via the phagocytosis of injured tissue (McLennan 1996; Mitchell et al. 1992; Papdimitriou et al. 1990). Recent evidence indicates that inflammatory cells may have additional effects within regenerating muscle after overt injury. Specifically, neutrophils have been suggested to both impair and aid muscle regeneration by injuring myotubes (Pizza et al. 2001) and activating satellite cells (Seale and Rudnicki 2000), respectively. Based on their ability to increase the number of MyoD+ cells and cause myoblast proliferation in vitro (Giurisato et al. 1998; Massimino et al. 1997; Merly et al. 1999), it has been suggested that macrophages,

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particularly rat ED2 macrophages, contribute to satellite cell activation and proliferation *in vivo*. Alternatively, rat ED1 macrophages, generally thought to be the macrophage cell type responsible for the phagocytosis of injured tissue (McLennan 1996), has recently been suggested to abrogate satellite cell activation and proliferation after traumatic injury (Kuschel et al. 2000). Because the different subpopulations of inflammatory cells may have opposing actions, it is difficult to predict if neutrophils, ED1 macrophages, and/or ED2 macrophages influence satellite cell activation after injurious exercise.

In the present study, we quantified muscle neutrophil and macrophage (ED1 and ED2) concentrations in rat solei after muscle injury induced by a single bout of downhill running. Because satellite cell activation is indicated by the up-regulation of myogenic regulatory factor MyoD (Koishi et al. 1995; McLoon et al. 1998; Tamaki et al. 2000), we also determined the number of MyoD+ cells to evaluate temporal relationships between satellite cell activation and changes in neutrophils, ED1 macrophages, and/or ED2 macrophages.

Methods

Animals

Male Wistar rats [$n=47$, body mass = 206 (13.9) g, mean (SD)] (Harlan Sprague Dawley; Indianapolis, Ind., USA) were housed individually, maintained on a 12:12-h light–dark cycle, fed and provided with water *ad libitum*. Procedures were approved by the Committee on Ethics in the Care and Use of Laboratory Animals at the University of Toledo.

Experimental treatment

Experimental rats completed an intermittent bout of treadmill exercise. The exercise protocol consisted of running (17 m/min) downhill (–16% grade) for 5 min followed by a 2-min rest interval (Armstrong et al. 1983; Ogilvie et al. 1988). The exercise–rest interval sequence was repeated a total of 18 times, resulting in an exercise duration of 90 min (Armstrong et al. 1983; Ogilvie et al. 1988). To encourage running, the hindlimbs of rats were stimulated with a soft bristled brush or paper towels. Rats were sacrificed using carbon dioxide at either 0 ($n=10$), 2 ($n=6$), 6 ($n=5$), 24 ($n=8$), 48 ($n=6$), or 72 h ($n=6$) post-exercise. Control rats ($n=6$) maintained normal cage activity.

The solei were excised, stapled to balsa wood coated with optimal cutting temperature medium (Fisher Scientific, Pittsburgh, Pa., USA), frozen in melting isopentane cooled in liquid nitrogen, and stored in isopentane at -70°C . The soleus muscle was used because previous investigators have reported that this muscle is injured after downhill running (Armstrong et al. 1983; Ogilvie et al. 1988; Smith et al. 2001) and because previous investigators have evaluated inflammatory cells in the solei after the hindlimb suspension reloading model of muscle injury (Frenette et al. 2000, 2002; Tidball et al. 1999).

Immunohistochemistry

Frozen cross sections (10 μm) were cut (International Equipment Company Minotome, Needham Heights, Mass., USA) from the mid-belly of the soleus, adhered to 0.04% chromium (III) potassium sulfate–0.4% gelatin-coated slides and stored at -20°C . Sections

were then air dried for 30 min and fixed in cold acetone (inflammatory cells) or in 4% paraformaldehyde (MyoD) at room temperature for 10 or 15 min, respectively. Sections were washed in 15 mM phosphate-buffered saline (PBS) and blocked with 3% bovine albumin, 5% Tween 20, and 2% gelatin for 30 min. The primary antibodies for neutrophils, ED1 macrophages, and ED2 macrophages, which were applied for 2 h at room temperature, were a mouse anti-rat neutrophil (HIS48, 1:25, PharMingen, Franklin Lake, N.J., USA), mouse anti-rat ED1 (1:100; Serotec, Oxford, England), and mouse anti-rat ED2 (1:100, Serotec), respectively. MyoD protein was identified using a mouse anti-human MyoD antibody (clone 5.8A, 1:50, Dako, Carpinteria, Calif., USA) (Tamaki et al. 2000), which was applied overnight at 4°C . Slides serving as negative controls received PBS instead of primary antibody. After incubation with the primary antibody, sections were washed in PBS and then incubated with either biotinylated goat anti-mouse IgM (1:200 Vector Laboratories, Burlingame, Calif., USA) (HIS48) or biotinylated horse anti-mouse IgG (1:200, Vector Laboratories) (ED1, ED2, and MyoD) for 30 min. After a wash with PBS, horseradish peroxidase-avidin D (1:1000; Vector Laboratories) was applied for 30 min (or 45 min for MyoD). Slides were then developed using 3-amino-9-ethylcarbozyle Vectastain ABC reagent kit (Vector Laboratories).

Sections were viewed with an Olympus light microscope (Olympus, Tokyo, Japan) using Nomarski optics. Inflammatory cells and MyoD+ cells were quantified in two entire sections for each muscle by manually counting the number of positive cells. The area of each muscle section was determined using a calibrated eyepiece counting grid. The volume of muscle sampled was calculated as the product of the cross sectional area of the section and the section thickness (10 μm). In addition, the number of myofibers possessing neutrophils or ED1 macrophages within their cytoplasm were counted and expressed as a percentage of the total number of myofibers within the cross section. Labeled cells within blood vessels or on the periphery of the section were not counted.

Indices of muscle injury

Muscle injury was quantified via hematoxylin–eosin staining of two muscle cross sections for each muscle. The number of injured fibers was determined by counting the number of myofibers that exhibited one or more of the following overt signs of injury: (1) pale cytoplasm (Fig. 1A); (2) small diameter and angular shaped myofiber (Fig. 1B); (3) centrally located nuclei (Fig. 1B), and (4) invasion of myofibers with cells (Fig. 1C) (Koh and Brooks 2001; Smith et al. 2001). The number of injured fibers was reported as a percentage of the total number of fibers within each cross section. Blood was also obtained by cardiac puncture and the serum was analyzed for creatine kinase activity using an enzymatic method (Sigma, St. Louis, Mo., USA).

Statistics

A one-way analysis of variance (ANOVA; SigmaStat 2.03, Sigma) was used to compare (to controls) groups of rats sacrificed at different time points after downhill running. Upon finding significance in the ANOVA ($P \leq 0.05$), a Newman-Keuls post-hoc test was used to locate differences. Data are reported as mean (standard error).

Results

Consistent with previous investigations (Armstrong et al. 1983; Ogilvie et al. 1988; Smith et al. 2001), our downhill running protocol caused injury to the soleus as indicated by the significant elevation in the percentage of fibers showing overt signs of injury (Fig. 2A) and by the elevated blood creatine kinase activity (Fig. 2B). Neutrophils (Fig. 3A) were significantly elevated 18-fold relative to

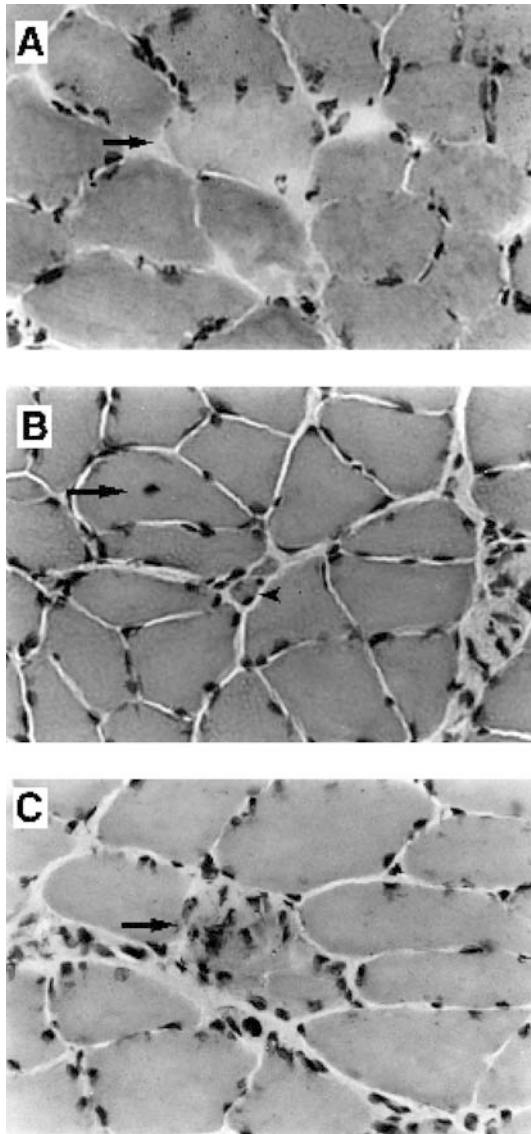


Fig. 1A, B Cross sections obtained post-exercise and stained with hematoxylin and eosin. **A** A myofiber with a pale cytoplasm (*arrow*; magnification 600 \times). **B** Small diameter and angular shaped myofibers (*arrowhead*) and a myofiber with a centrally located nucleus (*arrow*; magnification 600 \times). **C** A myofiber invaded by cells (*arrow*; magnification 600 \times). The number of myofibers in a cross section exhibiting the above characteristics were counted and used as a measure of muscle injury

controls at 24 h post-exercise. Although neutrophils were elevated by five-, six-, and ninefold at 2, 6, and 48 h post-exercise, respectively, neutrophils at these time points were not significantly different from levels observed in controls. ED1 macrophages (Fig. 3B) were significantly elevated four- and twofold at 24 and 48 h post-exercise, respectively. The percentage of fibers invaded by either neutrophils or ED1 macrophages followed the same pattern of significance as observed for neutrophil and ED1 macrophage concentrations, respectively (data not reported). Surprisingly, ED2 macrophages were not elevated during the 72 h of recovery from downhill running (Fig. 3C).

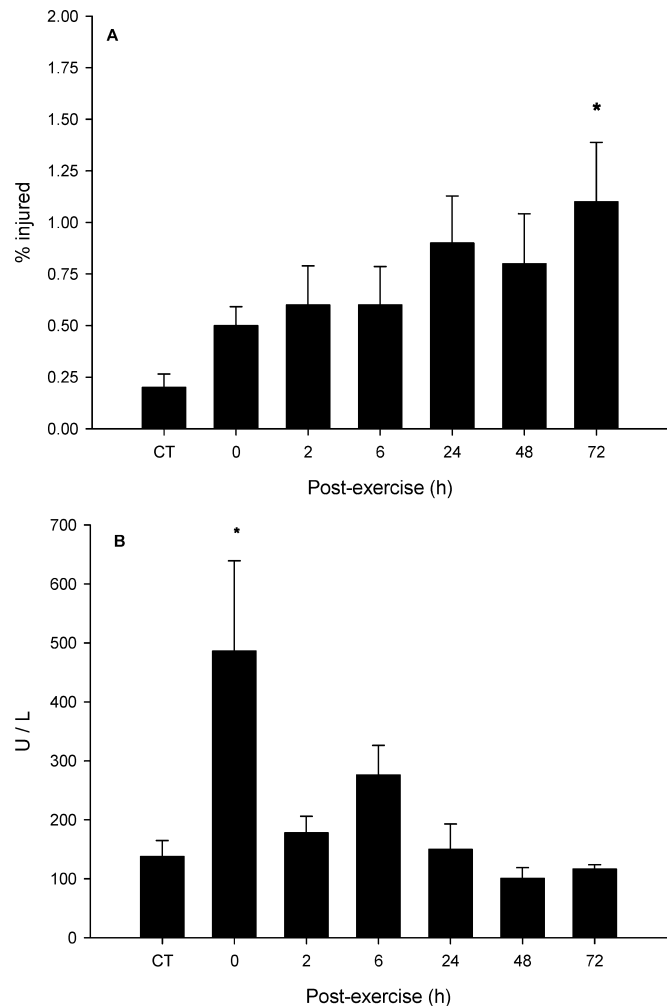


Fig. 2A, B Markers of muscle injury. **A** Percentage of injured fibers calculated as percentage of the total number of fibers that exhibited overt signs of injury as described in Methods and illustrated in Fig. 1. **B** Blood creatine kinase activity. * Significantly different relative to controls (CT)

MyoD⁺ cells in the soleus muscle of controls were located on the periphery of a few myofibers. On average, two MyoD⁺ cells in a cross section were observed in the soleus muscle of controls. After the downhill-running protocol, MyoD⁺ cells were predominantly found on the periphery of apparently normal myofibers (Fig. 4a) and in some instances, they were found in and/or around injured myofibers. Although the average number of MyoD⁺ cells in a cross section obtained at 0 h post-exercise was six, the number of MyoD⁺ cells at the post-exercise time points were not significantly different from levels observed in controls (Fig. 4b). The MyoD⁺ cells were presumed to be activated satellite cells.

Discussion

Novel observations of the present study were that a single bout of downhill running in rats elevated both muscle neutrophils and ED1 macrophages and resulted

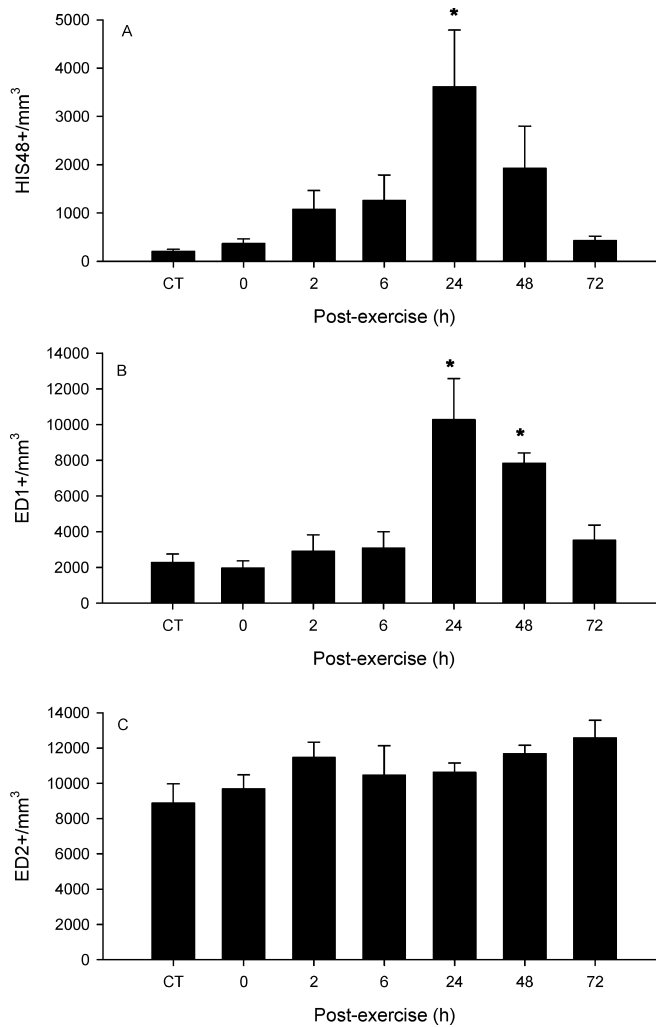


Fig. 3A–C Inflammatory cells. **A** Neutrophil concentrations. **B** ED1 macrophage concentrations. **C** ED2 macrophage concentrations. * Significantly different relative to CT

in non-significant changes in ED2 macrophages and MyoD⁺ cells. These observations demonstrate that the elevations in neutrophils and ED1 macrophages after a protocol of downhill running occur in the absence of change in MyoD⁺ cells, a marker of satellite cell activation (Koishi et al. 1995; McLoon et al. 1998; Tamaki et al. 2000).

The elevation in neutrophils in the present study is consistent with our previous observations after in situ lengthening contractions of the extensor digitorum longus (EDL) muscle in male mice (Pizza et al. 2002) and in contrast to a recent report by Lapointe et al. (2002). Lapointe et al. (2002) exposed young (50–70 g) female rats to in situ lengthening contractions of the EDL and quantified neutrophils via immunohistochemistry using an antibody against leukosialin (CD43, clone W3/13, Serotec), a membrane sialoglycoprotein found on several types of leukocytes. They concluded that neutrophils (CD43⁺ cells/mm³) were not significantly elevated during the hours to days after overt injury caused by lengthening contractions. Conflicting

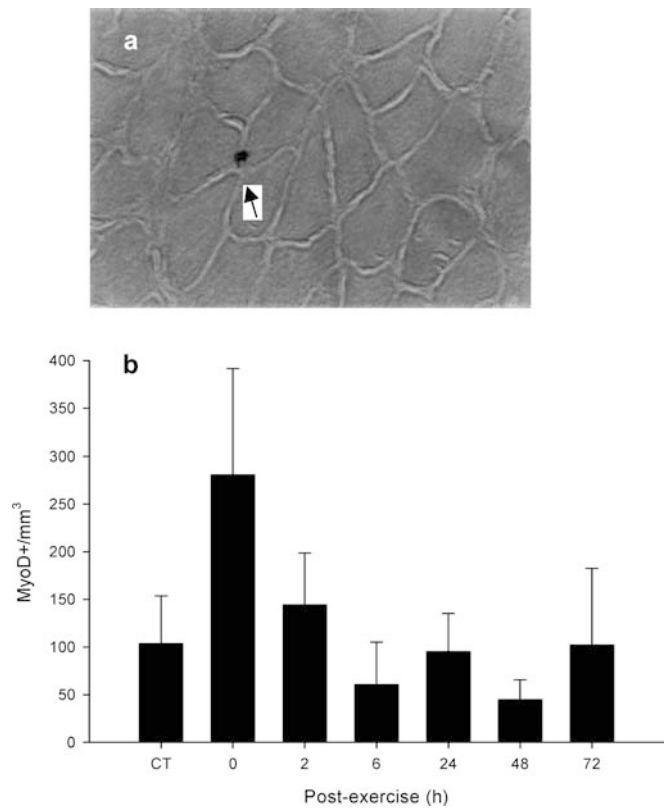


Fig. 4a, b MyoD⁺ cells. **a** Muscle cross section obtained at 0 h post-exercise and treated with an anti-MyoD antibody (magnification 400×), arrow indicates a MyoD⁺ cell. **b** Concentration of MyoD⁺ cells. The number of MyoD⁺ cells post-exercise were not significantly different from CT

neutrophil results between the present study and that of Lapointe et al. (2002) are not easily resolved but may be partially related to differences in the mode of exercise-induced muscle injury (downhill running and electrically stimulated lengthening contractions, respectively), differences in the mass (192–220 g and 50–70 g, respectively) and/or the gender (male and female, respectively) of the rats used (Tiidus 2001). In addition, because CD43 is expressed by other leukocytes and because it is known to be shed from neutrophils during activation (respiratory burst and degranulation) and adhesion to endothelial cells (Bazil and Strominger 1993; Lopez et al. 1998; Rieu et al. 1992), CD43 may not be an appropriate antibody for labeling neutrophils in skeletal muscle.

The functional significance of ED1 and ED2 macrophages in skeletal muscle has been inferred, largely based on their time course of accumulation and their distribution within injured skeletal muscle. ED1 macrophages, which are derived from blood monocytes, are the first subpopulation of macrophages to accumulate in skeletal muscle after injury induced by hindlimb suspension reloading (Frenette et al. 2000, 2002, Tidball et al. 1999). Because ED1 macrophages are known to invade muscle fibers after injury they are thought to be important in muscle repair by removing injured tissue via phagocytosis (McLennan 1996). The increase in ED1

macrophages at 24 and 48 h after downhill running in the present study is in agreement with the observations of Lapointe et al. (2002) after lengthening contractions. ED2 macrophages, the so-called resident macrophages, have been reported to accumulate in skeletal muscle, but not invade muscle fibers, 1–3 days post-injury (Frenette et al. 2000, 2002; Tidball et al. 1999). Based on cell culture studies, it has been suggested that ED2 macrophages contribute to muscle regeneration by causing satellite cell activation and myoblast proliferation (Massimino et al. 1997). Unexpectedly, ED2 macrophages were not elevated during 72 h of recovery from our downhill running protocol, an observation that is not consistent with the reported elevations in ED2 macrophages 48 h after lengthening contractions by Lapointe et al. (2002). One possible explanation for the lack of change in ED2 macrophages in the present study is that our downhill-running protocol may not have induced enough injury to the soleus to elicit an increase in ED2 macrophages. Further work is needed to identify the mechanisms for the accumulation of ED2 macrophages in injured skeletal muscle before the conflicting results can be reconciled.

The activation of satellite cells, a prerequisite for their subsequent proliferation and differentiation, is indicated by increased expression of the myogenic regulatory factors MyoD and/or Myf-5 that can transactivate muscle-specific gene expression (Cantini et al. 1995; Koishi et al. 1995; Seale and Rudnicki 2000). Studies have reported that MyoD (mRNA and/or protein) is up-regulated by overt muscle injury induced by muscle trauma (crush-, freeze-, or bupivacaine-induced injury) (Grounds et al. 1992; Koishi et al. 1995; McLoon et al. 1998) and by a single bout of weight-lifting exercise (Tamaki et al. 2000). Since previous investigators have demonstrated satellite-cell proliferation in soleus muscle 24 h after downhill (Darr and Schultz 1987) or level running (Jacobs et al. 1995), we hypothesized that the number of MyoD+ cells would be elevated 24–72 h following our downhill-running protocol. Contrary to our hypothesis, MyoD+ cells were not significantly elevated above control levels post-exercise (Fig. 4b). The lack of change in MyoD+ cells is consistent with recent qualitative observations of Smith et al. (2001) following a bout of downhill running (30 min, –16% grade, 15 m/min) in rat solei. The nonsignificant change in MyoD+ cells in the present study may indicate that either our downhill-running protocol did not induce sufficient injury to the soleus to cause satellite cell activation, that other myogenic regulatory factors (e.g., Myf-5) initiated satellite cell activation in the soleus after downhill running, and/or that the elevations in neutrophils and/or ED1 macrophages prevented satellite cell activation.

Although poorly understood, it has been suggested that the different subpopulations of inflammatory cells either enhance or inhibit satellite cell activation in vivo. Seale and Rudnicki (2000) have hypothesized that neutrophils contribute to the activation and proliferation of satellite cells via the interaction of the

neutrophil α_4 -integrin receptor with vascular cell adhesion molecule-1 expressed by myoblast and myotubes. Considering that neutrophils may release several factors (e.g., tumor necrosis factor - α , interleukin 1 β , and interferon) (Cassatella 1999) that have been reported to inhibit either myoblast proliferation and/or differentiation in vitro (Garrett 1992; Hawke and Garry 2001; Langen et al. 2001), it is possible that neutrophils negatively regulate satellite cells in vivo. In a similar fashion, ED1 macrophages have been suggested as negatively regulating satellite cells in vivo via their expression of allograft inflammatory factor-1, a reported inhibitor of cultured myoblast proliferation and differentiation (Kuschel et al. 2000). ED2 macrophages on the other hand have been reported to increase the number of MyoD positive myoblasts in vitro and are thought to be the subpopulation of rat macrophages that contribute to myogenesis in vivo (Massimino et al. 1997). Whether the in vitro observations on the influence of inflammatory cells and/or their derived products on satellite cells is applicable to exercise-induced muscle injury is unknown.

In conclusion, muscle injury induced by downhill running was associated with elevations in neutrophils and ED1 macrophages, but not ED2 macrophages in rat solei, during 72 h of recovery. The elevations in neutrophils and ED1 macrophages however, were not temporally associated with an increase in satellite cell activation, as indicated by a lack of change in MyoD+ cells.

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