Estrogen influences satellite cell activation and proliferation following downhill running in rats

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Enns DL, Tiidus PM. Estrogen influences satellite cell activation and proliferation following downhill running in rats. J Appl Physiol 104: 347–353, 2008. First published December 20, 2007; doi:10.1152/japplphysiol.00128.2007.—To investigate the influence of estrogen on postexercise muscle repair processes, we examined the effects of estrogen supplementation (0.25-mg pellet) on numbers of myofibers positive for markers of total, activated, and proliferating satellite cells in rat skeletal muscles 72 h following downhill running. Ovariectomized female rats (n = 44) were divided into four groups (n = 11 per group): sham (no estrogen) controls (SC); sham, exercised (SE); estrogen-supplemented controls (EC); and estrogen-supplemented, exercised (EE). After 8 days of estrogen exposure, animals were exposed to 90 min of treadmill running at 17 m/min (~13.5°). Seventy-two hours later, soleus and white vastus muscles were removed and immunostained for satellite cells using antibodies against paired box homeotic gene 7 (Pax7), activated myogenic differentiation factor D (MyoD), and 5-bromo-2'-deoxyuridine (BrdU) satellite cells. β-Glucuronidase activity was increased (P < 0.05) in both muscles following exercise; however, the postexercise elevations in enzyme activity were attenuated in the EE group compared with the SE group in the soleus (P < 0.05). Immunohistochemical analysis revealed that exercised groups displayed increased numbers of myofibers containing total, activated, and proliferating satellite cells compared with control groups (P < 0.05). Furthermore, greater numbers of fibers positive for markers of total, activated, and proliferating satellite cells were observed postexercise in EE animals compared with SE animals for both muscles (P < 0.05). The results demonstrate that estrogen may potentially influence postdamage repair of skeletal muscle through activation of satellite cells.

exercise; injury; skeletal muscle; repair

SATELLITE CELLS ARE THE MYOGENIC precursor cells of postnatal skeletal muscle (for review, see Ref. 13). Located on the periphery of the myofiber between the sarcolemma and the basal lamina, they normally remain in a nonproliferative quiescent state; however, in response to myofiber injury (14, 38) and hypertrophic signals (15) they reenter the cell cycle, where they proliferate and differentiate to provide the necessary precursors for skeletal muscle growth and repair (35).

Strenuous, unaccustomed exercise, or exercise involving lengthening contractions can result in injury to myofibers (3, 9). The sequence of events during muscle repair has been well characterized (4), and it includes an infiltration of inflammatory cells (e.g., neutrophils and macrophages) to the site of injury followed by activation of satellite cells to repair and replenish the injured tissue. Satellite cell activation and proliferation is mediated by a number of factors generated by both the injured tissue and the inflammatory cells (49). For example, nitric oxide (NO) (2) and insulin-like growth factor I (24) have been shown to activate satellite cells following muscle injury, and these factors may in turn be influenced by circulating levels of estrogen (29, 40).

Animal studies have consistently demonstrated that sex differences exist in response to resistive and eccentric exercise protocols (1, 19, 39, 46). These changes have been attributed to the effects of estrogen on skeletal muscle, because differences have been observed with hormonally manipulated animals (e.g., ovariectomy plus estrogen supplementation) as well as between males and females (1, 46). More specifically, studies from our laboratory (40, 46) and others (1, 19, 39) have revealed that sex and estrogen attenuate leukocyte infiltration and indirect indices of muscle damage (e.g., activity of the lysosomal enzyme, β-glucuronidase) following muscle injury. Estrogen has also been shown to inhibit inflammation and accelerate healing in a number of other tissues, including liver and nervous tissue (12, 52).

Although the influence of estrogen on postexercise inflammatory responses has been well characterized, less is known about the potential effects of estrogen on muscle repair processes. There is evidence to suggest that estrogen mediates skeletal muscle repair through activation of satellite cells. For example, Roth et al. (31) observed a greater increase in the number of satellite cells in the vastus lateralis muscles of women compared with men following 9 wk of resistance training. A study involving mdx mice, which lack a functional dystrophin-glycan complex and undergo repeated cycles of necrosis and repair, revealed that skeletal muscles of female mice demonstrated less myonecrosis and had a greater number of myofibers positive for the satellite cell marker neural cell adhesion molecule compared with male mice (32).

Our laboratory recently published a preliminary study that revealed an increase in the numbers of paired box homeotic gene 7 (Pax7)-positive satellite cells in soleus and white vastus muscles of estrogen-supplemented male rats compared with unsupplemented males 72 h after downhill running (47). However, because Pax7 labels satellite cells that are quiescent, activated, or proliferating (13, 36), it is not yet known which stages of satellite cell activation are influenced by estrogen. Therefore, a primary aim of this study was to verify and characterize the influence of estrogen on satellite cell activation, proliferation, and total numbers in different fiber types of rat skeletal muscle following downhill running. In addition to measuring total satellite cell numbers via Pax7 labeling, we also measured levels of myogenic differentiation factor D (MyoD), which is present primarily in activated satellite cells.
(13) and 5-bromo-2′-deoxyuridine (BrdU), which is taken up by proliferating satellite cells (14). Activity of the lysosomal acid hydrolase, β-glucuronidase, was used as an indirect marker of muscle damage (21). We hypothesized that J) total satellite cell numbers would be greater in estrogen-supplemented ovariectomized female rats compared with sham (un-supplemented) rats following exercise, and 2) by potentially mediating events upstream of satellite cell activation, estrogen would influence both activation and proliferation of satellite cells during postexercise repair of skeletal muscle.

METHODS

Animals. This study was approved by the Animal Care Committee at Wilfrid Laurier University, and all procedures were performed in accordance with the Canada Council on Animal Care. A total of 44 ovariectomized female Sprague-Dawley rats (Charles River Laboratories, LaSalle, QC) were used in this study. Ovaries were surgically removed at 9 wk of age, and all animals were 10 wk old (1 wk postovariectomy) on arrival in our laboratory. Animals were housed two per cage in an environmentally controlled environment with a standard 12:12-h light-dark cycle and allowed access to food (Tekland 22/5 Rodent Diet, Harland-Tekland, Madison, WI) and water ad libitum.

Experimental protocol. Following 1 wk of acclimation in the laboratory, animals were randomly subdivided into two groups: one group received estrogen supplementation via subcutaneous pellet implantation (0.25 mg 17β-estradiol, 21-day time-release pellet, Innovative Research of America, Sarasota, FL), while the other group received a sham procedure. Each of these groups was in turn subdivided into exercise and control (unexercised) groups. Therefore, four groups of animals were used in this experiment (n = 11 per group): sham (no estrogen) controls (SC); sham, exercised (SE); estrogen-supplemented controls (EC); and estrogen-supplemented, exercised (EE).

Pellet implantation and sham procedures were performed under aseptic conditions. Rats were anesthetized via inhaled isoflurane, and a small incision was made in the skinfolds of the neck. Blunt dissection and insertion of the forceps (1 cm from the site of incision using forceps. For the sham procedure, blunt dissection and insertion of the forceps were performed without pellet insertion. Following each procedure, the incision was sealed using one to two drops of Vetbond (3M, St. Paul, MN). Animals were returned to their cages and allowed to recover for 8 days before beginning the exercise protocol. This recovery time allowed the pellet-implanted groups to receive prolonged exposure to estrogen. Animals also received two 5-min familiarization sessions on the motorized rodent treadmill (Columbus Instruments, Columbus, OH) 3 and 4 days after the procedure.

During the exercise protocol, animals in the SE and EE groups were run downhill at a −13.5° grade at a speed of 17 m/min for a total of 90 min using the method described by Komulainen et al. (20). During the exercise, 5-min running bouts were interspersed with 2-min rest periods. This protocol is nonfatiguing and has been shown to elicit significant damage to soleus and white vastus muscles (3, 11).

Tissue collection. Seventy-two hours after completion of the exercise protocol, animals were injected with an overdose of pentobarbital sodium (55 mg/kg ip), and blood samples, soleus and superficial (white) portions of the vastus muscles were removed. These muscles were chosen for analysis because it has been suggested that satellite cell numbers may differ between fiber types, with slow-twitch fibers having more satellite cells than fast-twitch fibers (13). Nonexercised (control) animals were euthanized at the same time as the exercised animals. Sixty minutes before being killed, animals were injected with 50 mg/kg (ip) BrdU (Sigma-Aldrich, St. Louis, MO) dissolved in sterile physiological (0.9%) saline (38). BrdU is taken up by proliferating satellite cells, which incorporate the label into their DNA (14). Blood samples (~5 ml) were collected from the femoral artery, allowed to clot, and centrifuged at 3,000 g. The serum was removed and stored at −20°C until further analysis. Muscle samples were rinsed in physiological saline to remove excess blood, blotted dry, and trimmed of visible connective tissue. Samples used for immunohistochemical analysis were mounted on a specimen holder coated in optimal cutting temperature medium (Tissue-Tek, Torrance, CA) and frozen in isopentane previously cooled in liquid nitrogen. Tissue samples used for enzyme and protein analyses were immediately frozen in liquid nitrogen. All samples were stored at −20°C until analysis.

Serum analysis. Serum estrogen levels were determined in duplicate using a commercially available radioimmunoassay kit (Coat-a-Count TKE21, Inter Medico, Markham, ON). The test-retest variability of this procedure in our laboratory is 4–7%.

β-Glucuronidase activity. β-Glucuronidase activity was measured in soleus and white vastus muscles using the method of Barrett (5) as described by Koskimies et al. (21). Samples were assayed in triplicate at 420 nm. Activities were expressed as the amount of substrate (5 mM p-nitrophenyl-β-D-glucuronide, Sigma-Aldrich) hydrolyzed per amount of protein and incubation time. Protein was quantified according to the method of Lowry et al. (23).

Immunohistochemistry. Transverse serial sections of soleus and white vastus muscles were cut at 7 μm using a cryostat (Leica CM3050S) at −20°C. Sections were mounted on coated glass slides (Vectabond, Vector Laboratories, Burlington, ON, Canada), allowed to dry for 45 min, and stored at −20°C until staining. Sections were immunostained for markers of total (Pax7), activated (MyoD), and proliferating (BrdU) satellite cells. All samples were fixed in cold (4°C) acetic acid for 10 min. Endogenous peroxidases were inactivated by adding 0.6% hydrogen peroxide in absolute methanol, followed by three washes in 15 mM PBS (pH 7.6). Nonspecific sites were blocked for 30 min using 5% normal goat serum in PBS (containing 5% nonfat milk powder). For Pax7 immunostaining, slides were incubated with an antibody to Pax7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) for 40 min at a dilution of 1:20. For MyoD immunostaining, slides were incubated overnight at 4°C in an antibody to MyoD (Dako Canada, Mississauga, ON, Canada) at a dilution of 1:50. For BrdU immunostaining, slides were incubated overnight at 4°C in an antibody to BrdU (Vector Laboratories) at a dilution of 1:20. Negative controls were incubated in blocking solution for the same duration as each primary antibody. After three rinses in PBS, slides were developed using a commercially available kit (LSAB-2, Dako Canada) containing biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase. Satellite cells were visualized using either NovaRed (Pax7, Vector Laboratories) or diaminobenzidine with nickel (MyoD and BrdU, Vector Laboratories). Slides stained for Pax7 and MyoD were counterstained with hematoxylin (QS (Vector Laboratories) and mounted with permanent mounting medium (Vector Laboratories). Because the BrdU antibody recognizes BrdU that has been incorporated into DNA in a number of intracellular compartments in addition to satellite cells, slides stained with the anti-BrdU antibody were stained with an anti-laminin polyclonal antibody (Dako Canada, 1:500 dilution for 30 min) to ensure that BrdU-positive satellite cells located beneath the basal lamina and adjacent to the sarcolemma were clearly identified.

To quantify satellite cells, slides were placed under a light microscope (Leica DMLS) with the image projected onto a computer screen at a magnification of ×400 (×10 ocular and ×40 objective lens). Satellite cells were identified as ovoid dark-staining bodies located between the sarcolemma and the basal lamina (Fig. 1). Myofibers containing positive-staining central nuclei and structures located outside the satellite cell compartment were not counted. A total of 400 myofibers were counted from two individual sections for each muscle sample (200 fibers per section × 2 sections). The relative number of
myofibers positive for each marker was calculated as (number of positive fibers/400 × 100%). These measurements were considered to be representative of the relative number of positively stained satellite cells, because single myofibers containing more than one positively stained satellite cell were rarely observed (unpublished observations).

**Statistical analyses.** All data are presented as means ± SE. Serum estrogen levels, animal weights, and fiber-type comparisons were evaluated using a one-way ANOVA and a level of significance of P < 0.05. Where significance was found, differences between groups were evaluated using the Student-Newman-Keuls test.

**RESULTS**

**Serum estrogen levels and animal weights.** Estrogen-supplemented ovariectomized female rats had significantly higher serum estrogen levels than sham (unsupplemented) rats (240 ± 28 vs. 12 ± 1 pg/ml; P < 0.0001). Estrogen-supplemented rats also weighed significantly less than unsupplemented rats at time of death (262 ± 3 vs. 317 ± 3 g; P < 0.0001). This finding [i.e., that ovariectomized (sham) animals have greater body weights than estrogen-supplemented animals] is consistent with a number of previous studies in rodents that have utilized ovariectomy and estrogen replacement (28, 40, 47).

**β-Glucuronidase activity.** β-Glucuronidase activity (Table 1) was measured to provide us with an indirect assessment of satellite cells. Because single myofibers containing more than one positively stained satellite cell were rarely observed (unpublished observations), these measurements were considered to be representative of the relative number of positively stained satellite cells, because single myofibers containing more than one positively stained satellite cell were rarely observed (unpublished observations).

**DISCUSSION**

This study expanded on preliminary work from our laboratory that demonstrated an increase in satellite cell numbers 72 h postexercise in estrogen-supplemented male rats compared with unsupplemented rats (47). A novel finding...
of this follow-up study was that in addition to an increase in total satellite cell numbers, we also observed increases in the number of myofibers staining positively for activated and proliferating satellite cells in female ovariectomized rats supplemented with estrogen 72 h following a similar downhill running protocol. These findings confirm that not only does estrogen play a role in muscle repair processes by influencing activation and proliferation of satellite cells but also it appears that estrogen may act upstream of satellite cell activation through mechanisms which are at this time are still unknown.

Fig. 2. Effects of estrogen supplementation on relative numbers of myofibers expressing total (Pax7; A), activated (MyoD; B), and proliferating (BrdU; C) satellite cell markers in rat soleus muscle 72 h following downhill running. Values are means ± SE; n = 11 rats per group. *P < 0.05 compared with control. #P < 0.05 compared with sham.

Fig. 3. Effects of estrogen supplementation on relative numbers of myofibers expressing total (Pax7; A), activated (MyoD; B), and proliferating (BrdU; C) satellite cell markers in rat white vastus muscle 72 h following downhill running. Values are means ± SE; n = 11 rats per group. *P < 0.05 compared with control. #P < 0.05 compared with sham.
Muscle β-glucuronidase activity was measured as an indirect assessment of muscle injury induced by our experimental protocol. This marker has been shown to be a reliable indicator of exercise-induced muscle injury (19, 33). Consistent with our findings, Komulainen et al. (19) reported that downhill running-induced increases in β-glucuronidase activity were attenuated in the hindlimb muscles of female rats relative to male rats. Although some authors have suggested that body mass may be related to muscle damage (18), other studies, including the present study, observed no relationship between body mass and muscle damage, as assessed by β-glucuronidase activity, after downhill running (19).

It is possible that estrogen may have had a protective effect on muscle damage by directly influencing contractile function. Recently, Moran et al. (28) demonstrated that ovariectomy diminished skeletal muscle contractility in adult female mice, and they suggested that the loss was due to a lack of availability of strong-binding myosin during contraction. In a follow-up study by the same laboratory, estrogen replacement restored contractile function and the availability of strong-binding myosin; however, estrogen was not effective in protecting the muscle against eccentric contraction-induced injury (27). Although we did not quantify postexercise structural changes in our muscle samples, the influence of estrogen on the contractile apparatus may in part explain why β-glucuronidase activities were attenuated in the estrogen-supplemented group.

In agreement with our laboratory’s preliminary study (47), we observed an increase in the number of fibers containing Pax7-positive satellite cells 72 h following downhill running in both soleus and white vastus muscles. Furthermore, the exercise-induced increase in the number of fibers containing activated (i.e., MyoD-positive) and proliferating (i.e., BrdU-incorporated) satellite cells was greater in the estrogen-supplemented group compared with the sham group, thus confirming our hypothesis. Based on our findings, it is possible that estrogen may influence muscle repair by mediating events upstream of satellite cell activation because no differences were observed between the numbers of fibers containing activated and proliferating satellite cells.

Surprisingly, a difference in the number of Pax7-positive fibers was evident between estrogen-supplemented and sham groups in the control condition in the white vastus but not in the soleus. It is not known why these differences were observed, because similar trends were not observed in our laboratory’s preliminary study (47). It is possible that sex differences may have influenced the trends, because the present study utilized ovariectomized female rats, whereas male rats (with or without estrogen supplementation) were used in the earlier study.

In this study, the numbers of fibers that stained positively for markers of total (Pax7) and activated (MyoD) satellite cells were greater in the soleus compared with the white vastus in control (Pax7) and exercised (Pax7, MyoD) conditions. These observations are similar to those of Schultz et al. (34), who noted a greater percentage of Pax7- and MyoD-labeled nuclei in the soleus (an oxidative muscle) compared with the extensor digitorum longus (a glycolytic muscle). It is not known why we did not observe a greater number of MyoD-positive myofibers in the soleus compared with the white vastus in our control conditions. It is possible that the difference in the number of myofibers counted [400 myofibers per muscle in our study vs. >1,000 myofibers per muscle in the Schultz et al. (34) study] may have accounted for some of the differences. It should also be noted that not all studies have demonstrated fiber-type differences with respect to the number of satellite cells present in control and exercised muscles (16, 47).

In this study, we chose to evaluate satellite cell activity at a single time point (72 h) following lengthening exercise. This time point was chosen because we wanted to use a time point where total, activated, and proliferating satellite cell numbers would be at peak levels. The generally accepted sequence of events during muscle injury and repair suggests that most inflammation and satellite cell activation occur within the first 72 h after injury (3, 13). To verify that 72 h postexercise was the ideal time point to study satellite cell activation and proliferation, we performed a pilot study to determine the time course of Pax7 and MyoD expression and BrdU incorporation in satellite cells of skeletal muscles of ovari-intact female rats following a similar downhill running protocol (8). In this pilot study, animals were killed 1, 2, 3, and 4 days postexercise. The number of fibres staining positively for Pax7-labeled satellite cells steadily increased 1–3 days postexercise, whereas the number of fibres containing BrdU-positive satellite cells peaked 2 and 3 days postexercise, whereas the number of fibres containing BrdU-labeled satellite cells reached peak levels 3 days postexercise. These findings are consistent with the postinjury time course of satellite cell activation and proliferation reported by others (10, 14), and they confirm the validity of the 72-h postexercise time point.

The mechanisms by which estrogen may potentially influence postinjury satellite cell activation, proliferation, and total numbers are not known. Sex differences in response to exercise-induced muscle injury have been well documented in both humans and animals (7, 44). Attenuated levels of creatine kinase activity (1), neutrophil infiltration (46), and ultrastructural disruptions (19) are observed in female rodents relative to males. These changes have been attributed specifically to the influence of estrogen, because removal of estrogen (i.e., via ovariectomy) also removes this effect (1, 48).

Although postexercise changes in leukocyte infiltration were not measured in this study, previous work from our laboratory has consistently shown that neutrophil infiltration is attenuated with estrogen supplementation following exercise and muscle injury (40, 47). Although the mechanisms by which estrogen may influence neutrophil infiltration remain elusive, the ability of estrogen to act as a membrane stabilizer and antioxidant may enhance membrane fluidity and limit sarcoclemmal disruption (45). It is possible that the protective role of estrogen on sarcosomal disruption and subsequent inflammatory cell infiltration may paradoxically be detrimental to satellite cell activation. Further studies are needed to clarify the role of estrogen as both a protective reagent and as a potential signaling molecule following muscle injury.

Macrophages also play a role in recovery of skeletal muscle following exercise or myotrauma. Although macrophages are mainly known as scavengers of tissue debris following muscle injury, they are also involved in the regeneration process (26). In vitro studies have found increases in the numbers of activated (MyoD-positive) and proliferating (BrdU-positive) satellite cells in myoblasts when macrophages were added to the culture (25, 26). Furthermore, Tidball and Wehling-Henricks (43) recently demonstrated that the number of activated satel-
lite cells (as indicated by numbers of MyoD-positive satellite cells) decreased when macrophages were selectively depleted during muscle reloading after a period of hindlimb suspension in mice. With respect to sex differences, St. Pierre Schneider et al. (39) reported that macrophage infiltration was delayed in female mice relative to male mice after lengthening contractions. Hence, it is possible that estrogen may influence satellite cell activation and hence muscle repair indirectly through its effects on specific macrophage populations.

Studies of muscle injury in animal models have also suggested that NO may be a critical effector of estrogen-mediated protection, and this process is potentially regulated through receptor- and/or non-receptor-mediated events. Estrogen has been shown to augment NO release and activate NO synthase (NOS) isoforms in a number of tissues, and in so doing, it may influence muscle damage and recovery processes (6, 29). In skeletal muscle, NO is produced by the NOS I isoform at the sarcolemma, and nonspecific inhibition of NOS with N-nitroarginine methyl ester before muscle injury has been shown to prevent satellite cell activation (2). In addition, the release and localization of hepatocyte growth factor, a known regulator of satellite cell activation (41), is an NO-dependent process during conditions of muscle fiber stretch and injury (42).

It is also possible that, in addition to NOS-related mechanisms, the estrogen-related augmentation in the number of fibers positive for the various satellite cell markers is a result of sparing from damage and inflammation. The presence of fewer inflammatory cells in the estrogen-supplemented groups may have allowed for a more rapid response during the phase of repair immediately following inflammatory cell infiltration, which corresponds with the 72-h postexercise time point used in this study.

Estrogen may also potentially exert its influence through receptor-mediated events. Mammalian skeletal muscle expresses both α- and β-type estrogen receptors (ERs) (50), and ERs have recently been identified in satellite cells of pig skeletal muscles (17). ERs have also been shown to be upregulated with exercise and training in animals (22) and humans (51). ERs, through estrogen binding, modulate a number of diverse intracellular signaling pathways, including the phosphatidylinositol 3-kinase/protein kinase B (Akt) pathway, which stimulates growth and protein synthesis (30, 37). For example, Sitnick et al. (37) demonstrated that ovariectomy prevented recovery of atrophied skeletal muscle in rats following a period of unloading and decreased phosphorylation of Akt-p70S6K signaling proteins. Furthermore, ER inhibition with a receptor antagonist (ICI-182780) reversed activation of the Akt pathway and increased apoptosis in cardiomyocytes of ovariecotomized female mice compared with estrogen-supplemented mice (30). The possibility that ER binding may signal other downstream events (which may in turn influence satellite cell activation) is intriguing and worthy of future investigation.

In conclusion, this study has demonstrated that estrogen supplementation influences activation and proliferation of satellite cells following lengthening contraction-induced muscle injury in rats. Although the specific mechanism(s) underlying estrogenic influence are still unclear, a number of possibilities exist, including both receptor- and non-receptor-mediated events. Understanding how estrogen exerts its protective effects while simultaneously influencing satellite cell activation will greatly contribute to our understanding of the molecular events underlying muscle injury and repair.

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GRANTS

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