Mechanical loading and injury induce human myotubes to release neutrophil chemoattractants

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Tsivitse, Susan K., Eleni Mylona, Jennifer M. Peterson, William T. Gunning, and Francis X. Pizza. Mechanical loading and injury induce human myotubes to release neutrophil chemoattractants. Am J Physiol Cell Physiol 288: C721–C729, 2005. First published November 17, 2004; doi:10.1152/ajpcell.00237.2004.—The purpose of this study was to 1) test the hypothesis that skeletal muscle cells (myotubes) after mechanical loading and/or injury are a source of soluble factors that promote neutrophil chemotaxis and superoxide anion (O2·−) production and 2) determine whether mechanical loading and/or injury causes myotubes to release cytokines that are known to influence neutrophil responses [tumor necrosis factor-α (TNF-α), IL-8, and transforming growth factor-β1 (TGF-β1)]. Human myotubes were grown in culture and exposed to either a cyclic strain (0, 5, 10, 20, or 30% strain) or a scrape injury protocol. Protocols of 5, 10, and 20% strain did not cause injury, whereas 30% strain and scrape injury caused a modest and a high degree of injury, respectively. Conditioned media from strained myotubes promoted chemotaxis of human blood neutrophils and primed them for O2·− production in a manner that was dependent on a threshold of strain and independent from injury. Neutrophil chemotaxis, but not priming, progressively increased with higher magnitudes of strain. Conditioned media only from scrape-injured myotubes increased O2·− production from neutrophils. Concentrations of IL-8 and total TGF-β1 in conditioned media were reduced by mechanical loading, whereas TNF-α and active TGF-β1 concentrations were unaffected. In conclusion, skeletal muscle cells after mechanical loading and injury are an important source of soluble factors that differentially influence neutrophil chemotaxis and the stages of neutrophil-derived reactive oxygen species production. Neutrophil responses elicited by mechanical loading, however, did not parallel changes in the release of IL-8, TGF-β1, or TNF-α from skeletal muscle cells.

Skeletal muscle contains several cell types (e.g., skeletal muscle cells, endothelial cells, fibroblasts, and resident macrophages) that can produce factors that influence neutrophil chemotaxis and reactive oxygen species (ROS) production. Because of this complexity, the cellular source of factors that influence neutrophil responses cannot be identified with in vivo mechanical loading and/or injury models. Previous investigators, however, demonstrated that cultured skeletal muscle cells can produce numerous factors that are known to influence neutrophils. Thus mechanical loading and traumatic injury to cultured skeletal muscle serve as an ideal paradigm for determining the contribution of skeletal muscle cells to neutrophil chemotaxis and ROS production after exercise and muscle trauma.

Although neutrophil-derived ROS are essential for the phagocytosis of pathogens and injured tissue, the release of ROS from neutrophils is known to cause injury to a variety of cell types, including skeletal muscle cells. Whether skeletal muscle cells are the source of primers and/or activators for neutrophil-derived ROS production after mechanical loading and/or injury, however, is unknown.

The primary purpose of the present study was to develop a cell culture model that determines the contribution of differentiated skeletal muscle cells (myotubes) to neutrophil responses after mechanical loading and injury. A secondary purpose was to determine whether mechanical loading and/or injury causes myotubes to release cytokines [tumor necrosis factor-α (TNF-α), IL-8, and transforming growth factor-β1 (TGF-β1)] that are known to influence neutrophil chemotaxis and ROS production. We hypothesized that myotubes release factors after mechanical loading that cause neutrophil chemotaxis and that these factors for ROS production in a manner that is dependent on the degree of strain.
and independent from muscle injury. The release of factors that fully activate neutrophils for ROS production, we hypothesized, would occur only after traumatic and strain-induced myotube injury. Support for our hypotheses would lend credence to our working model, in which the local environment dictates the concentration and the functional outcome of neutrophils in injured and noninjured skeletal muscle after exercise.

MATERIALS AND METHODS

Muscle cell culture. Human myoblasts were obtained from a 17-year-old female donor and were negative for mycoplasma, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus (BioWhittaker, Walkersville, MD). Myoblasts were seeded (10,000 cells/cm²) in collagen I-coated tissue culture plates (6 well) that had a flexible, flat bottom (Flexcell International, Hillsborough, NC). After myoblasts proliferated to ~90% confluence in growth medium (BioWhittaker), myoblasts were induced to differentiate into myotubes by replacing the growth medium with DMEM (Sigma, St. Louis, MO) supplemented with 2% heat-inactivated fetal bovine serum (Sigma), 2 ng/ml EGF, 50 µg/ml gentamicin, and 50 µg/ml amphotericin-B (BioWhittaker). On the fifth day, myotubes were experimentally treated.

Treatment of myotubes. Myotubes were washed twice with HBSS followed by the addition of 3 ml of DMEM supplemented with 50 µg/ml gentamicin and 50 µg/ml amphotericin-B (basal medium). Myotubes were then exposed to mechanical strain, traumatic injury, or a control condition.

Myotubes were strained for 2 h in a humidified 5% CO₂ atmosphere with a vacuum-based system (Flexercell Strain Unit FX 4000; Flexcell International). Specifically, the application of cyclic (0.25 Hz) negative pressure caused the membrane of the tissue culture plate to undergo 5, 10, 20, or 30% maximal elongation. A rubber stopper (Flexcell International) was applied to control wells to prevent strain of myotubes. Five hours after the protocol was initiated, conditioned media were collected, pooled, and stored at 80°C (conditioned media).

Myotube injury. Myotube injury was quantified by measuring lactate dehydrogenase (LDH) in conditioned media according to the manufacturer’s instructions (Promega, Madison, WI). Incubating myotubes with 0.5% Triton X-100 solution for 45 min induced total LDH release. An injury index was calculated using the following equation: 

\[
\text{injury index} = \frac{[\varepsilon - b] - (t - b)}{\varepsilon - b}
\]

where \(\varepsilon\) is LDH release from myotubes exposed to strain or scrape injury, \(b\) is LDH release from control myotubes, and \(t\) is total release from Triton X-100-treated myotubes.

Transmission electron microscopy was performed to assess myotube membrane integrity. 5 h after the 10 and 30% strain protocols were initiated, briefly, myotubes were washed in 0.1 M cacodylate buffer (pH 7.4) and then fixed in 3% glutaraldehyde-1% lanthanaum chloride. Primary fixation was performed at room temperature for 2 min, followed by a 2-h incubation at 4°C. After being washed in cacodylate buffer, myotubes were immersed in 2% osmium tetroxide-1% lanthanum chloride at 4°C for 1 h and rinsed with 2.4,6-collidine-1% lanthanum chloride. Dehydration was accomplished by immersing monolayers in a series of increasing-purity ethanol solutions. Acetone was briefly used as the transitional fluid, followed by a 15-min immersion in 50% Spurr’s embedding medium and an overnight infiltration in 100% Spurr’s resin at 80°C. Monolayers were then removed from the plates and cut into smaller pieces, and thin sections (~900 Å) were obtained with a diamond knife. Monolayers were not stained with uranyl acetate and lead citrate to enhance lanthanaum visualization. Observations were made with a CM10 transmission electron microscope (Philips) at an accelerating voltage of 80 kV.

Neutrophil isolation. Heparinized venous blood was obtained from healthy volunteers after written informed consent was obtained. Heparinized blood was layered over a density gradient (1-step Polymorph; Accurate Chemical, Westbury, NY) and centrifuged to isolate neutrophils from mononuclear and red blood cells as previously described (31, 39). Neutrophils were then suspended in either basal medium or conditioned medium. The isolation procedures routinely yielded >97% neutrophils with cell viability >98% as determined by Trypan blue exclusion. After isolation procedures, neutrophils were used immediately for a neutrophil assay. Neutrophils collected from each donor were used only once for a given neutrophil assay.

Neutrophil chemotaxis. Neutrophil chemotaxis was measured in a 48-well chamber (Neuro Probe, Gaithersburg, MD). The lower wells of the chamber were loaded in quadruplicate with fMLP (10⁻⁷ M; Sigma), basal medium, conditioned medium, recombinant human (rh)IL-8 (BD Biosciences, San Diego, CA), or human (h)TGF-β1 (derived from human platelets; R&D Systems). All media were supplemented with 1% bovine serum albumin. A 3-µm-pore polivynylpyrrolidone-free polycarbonate filter (Neuro Probe) was placed between the upper and lower chambers. Neutrophils, suspended in basal medium (3.2 × 10⁶/ml), were added to the upper wells, and the chamber was placed in a humidified environment (5% CO₂ and 37°C) for 30 min. Nonmigrated neutrophils were removed from the top surface of the filter by washing with phosphate-buffered saline and by scraping against a rubber edge. Migrated neutrophils were fixed in absolute methanol and visualized with Wright-Giemsa stain (Fisher Scientific, Pittsburgh, PA). The total number of neutrophils in 20 high-power fields (×1000) per well was counted and averaged for each subject. Partially migrated neutrophils and contaminating leukocytes were not counted. Neutrophil chemotaxis was expressed as a chemotactic index with the following equation: 

\[

c = \frac{[\text{conditioned medium} - \text{basal medium}] / [\text{fMLP} - \text{basal medium}] \times 100}
\]

The inclusion of fMLP, a potent chemottractant for neutrophils, served as a positive control in the equation.

A checkerboard analysis using 30% strain medium was performed to ensure that the observed responses were attributable to neutrophil chemotaxis as opposed to chemokinesis (random migration) (1). Briefly, the upper wells of the chemotaxis chamber were loaded with neutrophils suspended in undiluted 30% strain medium, half-diluted 30% strain medium, or basal medium. Neutrophils were allowed to migrate for 30 min toward undiluted 30% strain medium, half-diluted 30% strain medium, or basal medium, which was loaded into the lower wells of the chamber. Neutrophil chemotaxis was then determined as described above.

Neutrophil-derived O₂⁻. The ability of conditioned media to prime and to activate neutrophils for O₂⁻ production was quantified with the cytochrome c assay (2, 29). Neutrophil priming was evaluated by suspending neutrophils (3.5 × 10⁶/ml) in basal medium, conditioned media, or LPS (Escherichia coli 0127:B8, 100 mM) in basal medium for 2 h and then assaying for O₂⁻ production in the presence of fMLP (2.5 × 10⁻⁶ M) after a 10-min incubation. Activation of O₂⁻ production was assessed by suspending neutrophils (3.7 × 10⁶/ml) in either basal or conditioned medium and then pipetting them into polypropylene tubes or fibronectin-coated wells (2 µg/well). The O₂⁻ assay consisted of running two sets of triplicate polypropylene tubes or fibronectin-coated wells. SOD (90 µg/ml, bovine erythrocytes; Sigma) was added to one set of tubes or wells, and an equal volume of HBSS was added to the other set. Ferricytochrome c (80 µM, horse heart; Sigma) was added to all tubes or wells, and the samples were then incubated at 37°C in a shaking water bath or a plate reader (SpectraMAX 190; Molecular Devices). For evaluation of the ability of conditioned media to increase O₂⁻ production for neutrophil...
neutrophils in suspension, catalase (1,000 U/ml; Sigma) and S-ethylisothiourea (a nitric oxide synthase inhibitor, 1,000 μM; Sigma) were added to all tubes to prevent the reoxidation of ferrocytochrome c by hydrogen peroxide and nitric oxide, respectively (48, 51). Catalase and S-ethylisothiourea were not added to fibronectin-coated wells because 1) we recently reported (31) that human neutrophils do not produce nitric oxide via nitric oxide synthase and 2) pilot experiments revealed that the inclusion of catalase in conditioned media did not improve our detection of O$_2^•$ when neutrophils were adherent to fibronectin, an observation that was in contrast to pilot experiments using neutrophil suspensions. The optical density of the supernatant or well was read spectrophotometrically at 550 nm. The quantity of O$_2^•$ produced was calculated from the difference in the mean absorbance of tubes or wells that were without SOD from those with SOD and by using the extinction coefficient for ferrocytochrome c (29.5 mM/cm). A neutrophil count was performed on the final cell preparation, and O$_2^•$ production was expressed as nanomoles of O$_2^•$ per 5 × 10^5 neutrophils.

Cytokines. IL-8 (BD Biosciences), TGF-β1 (Promega), and TNF-α (BD Biosciences) concentrations in conditioned media were quantified with ELISA kits according to the manufacturer’s instructions (n = 5–6 wells/condition). The concentration of active TGF-β1 was determined by omitting the acidification and neutralization steps as recommended by the manufacturer.

Statistics. Separate analysis of variance was used to evaluate the influence of mechanical strain and traumatic injury on neutrophil chemotaxis, ROS production, and cytokine concentrations in conditioned media. Neuman-Keuls post hoc test was used when a significant F ratio was observed (P ≤ 0.05). Data are reported as means ± SE.

RESULTS

Myotube injury. The injury index was not significantly elevated (relative to zero) after 5, 10, or 20% strain (Fig. 1). The 30% strain protocol, however, resulted in a significant increase in the injury index relative to all other strain magnitudes. As expected, scrape injury caused greater damage than 30% strain.

Electron microscopy observations of control cultures revealed that lanthanum was restricted to the extracellular surface of myotube membranes (Fig. 2). In contrast, myotubes exposed to 30% strain demonstrated cytoskeletal disruptions, diffuse appearance of lanthanum in the cytoplasm (Fig. 3), and numerous membrane blebs (Fig. 4). None of these hallmark signs of injury was apparent in myotubes exposed to 10% strain. Localization of lanthanum to cytoplasmic vacuoles, however, was observed in myotubes exposed to 10% strain.

Fig. 1. Myotube injury index after mechanical strain and scrape (SCP) injury. Lactate dehydrogenase release from individual wells (n = 11 or 12 per condition), including control and total release wells, was determined and used in the calculation of the injury index (see MATERIALS AND METHODS). *Significantly different from zero; #significantly different from 30% strain.

Fig. 2. Control human myotube. The myotube membrane has prevented the electron-dense metal lanthanum (filled arrows) from penetrating into the cytoplasm. Limited visualization of a few mitochondria (asterisks) and the alignment of cytoskeletal proteins (open arrows) is allowed by osmium tetroxide. Bar, 0.5 μm.

Fig. 3. Human myotube after 30% strain. Membrane rupture, indicated by discontinuities of the membrane (open arrows), has allowed for diffusion of lanthanum (arrowheads) into the cytoplasm. Muscle injury is also indicated by cytoskeletal disruptions (asterisk). Bar, 0.5 μm.
(Fig. 5) but not 30% strain. The localized appearance of lanthanum may be attributable to increased endocytosis to reseal small membrane lesions (14, 49) and/or to the transport of lanthanum into the cytoplasm (41).

Neutrophil chemotaxis. Conditioned medium from 5% strained myotubes failed to cause neutrophil chemotaxis relative to control medium (Fig. 6). Chemotaxis indexes, however, were significantly increased by 10, 20, and 30% strain and scrape injury relative to controls. Chemotaxis for 30% strain was also significantly higher relative to 10% strain. Checkerboard analysis confirmed that the observed responses were attributable to chemotaxis and not chemokinesis (Table 1).

Neutrophil priming. Conditioned medium from myotubes exposed to 5% strain did not prime neutrophils for $O_2^{-}$ production (Fig. 7). A significant priming effect, however, was observed for 10, 20, and 30% strain. The priming effect for 10, 20, and 30% strain was 96% of priming induced by the positive control LPS, indicating the robust nature of these strain magnitudes on neutrophil priming. The priming induced by scrape injury was 105% of the priming induced by LPS. Unexpectedly, conditioned medium from control myotubes caused a modest, but statistically significant, priming effect relative to basal medium.

Neutrophil $O_2^{-}$ production. We first tested whether injured myotubes release factors that increase $O_2^{-}$ production from unprimed neutrophils in suspension. Scrape-injured myotubes, but not strain-injured myotubes ($P = 0.10$), released factors that significantly increased $O_2^{-}$ production from neutrophils in suspension (Fig. 8). Conditioned medium from scrape-injured myotubes elicited levels of $O_2^{-}$ that were 76 and 88% of the levels elicited by the positive control fMLP at 10 and 60 min of incubation, respectively. Levels of $O_2^{-}$ elicited by conditioned medium from strain-injured myotubes were 64 and 88% of the values obtained in fMLP-stimulated neutrophils at 10 and 60 min of incubation, respectively. Conditioned control medium elicited similar $O_2^{-}$ production relative to basal medium.

We speculated that the inability of 30% strain medium to increase $O_2^{-}$ production from unprimed neutrophils was related to a suboptimal concentration of unknown activators in the medium. To test this hypothesis, control and 30% strain media were concentrated 10-fold by centrifugal filtration (Centriplus, 30-kDa cutoff; Millipore). Contrary to our hypothesis, concentrated 30% strain medium and concentrated control medium elicited similar $O_2^{-}$ production from neutrophils during 60 min of incubation ($n = 6$; data not reported).

We next tested whether neutrophil priming is a prerequisite for 30% strain medium to increase $O_2^{-}$ production from neutrophils in suspension. For these experiments, neutrophils were primed with unconcentrated 30% strain medium for 2 h and then assayed for their ability to produce $O_2^{-}$ after a 10-min exposure to either concentrated control or concentrated 30% strain medium. These experiments ($n = 6$) also failed to demonstrate an activating effect for 30% strain medium (data not reported).

Because previous investigators reported that the kinetics and the magnitude of ROS production elicited by some cytokines [e.g., TNF-$\alpha$ and granulocyte-macrophage colony-stimulating factor (GM-CSF)] is enhanced when neutrophils are adherent...
to the extracellular matrix protein fibronectin (12, 34–36), we also assessed the ability of conditioned media from strained myotubes to increase $\text{O}_2$ from neutrophils adherent to fibronectin. Conditioned media from myotubes exposed to 5, 10, 20, and 30% strain failed to increase $\text{O}_2$ from neutrophils adherent to fibronectin relative to control medium (Fig. 9).

Cytokines. The concentration of IL-8 in conditioned media was significantly reduced after 10 and 30% strain and scrape injury relative to control cultures (Fig. 10A). The concentration of TNF-$\alpha$ in conditioned media was not influenced by mechanical strain but was significantly elevated after scrape injury relative to control cultures (Fig. 10B). Total TGF-$\beta 1$ concentrations in conditioned media were significantly lower after 5 and 10% strain and were higher after scrape injury relative to control cultures (Fig. 10C). The active form of TGF-$\beta 1$ remained at control levels after mechanical strain and scrape injury (Fig. 10D).

Cytokine-induced neutrophil chemotaxis. To evaluate the potential contribution of IL-8 and TGF-$\beta 1$ to the observed neutrophil chemotaxis after mechanical strain, we assessed neutrophil chemotaxis to rhIL-8 or hTGF-$\beta 1$ by using

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Values are means ± SE chemotaxis index; $n = 3$. Lower wells of the chemotaxis chamber were loaded with basal medium (DMEM), DMEM-30% strain medium (1:1; 30% diluted), or 30% strain medium (30% undiluted). Upper wells contained neutrophils ($3.2 \times 10^6$ cells/ml) suspended in DMEM or 30% diluted or 30% undiluted. NA, chemotaxis for DMEM in the upper and lower wells was used in the calculation of the chemotaxis index as described in MATERIALS AND METHODS.

Fig. 6. Neutrophil chemotaxis. A: chemotaxis index for conditioned media obtained from control cultures (CT; $n = 12$) and from myotubes exposed to 5% ($n = 12$), 10% ($n = 12$), 20% ($n = 10$), and 30% ($n = 12$) strain and to a scrape injury protocol (SCP; $n = 12$). *Significantly different from CT; #significantly different from 10% strain. The mean number of neutrophils per high-powered field (HPF) for $N$-formylmethionyl-leucyl-phenylalanine (fMLP) and basal medium for all subjects was 75 and 9, respectively. B: mean number of neutrophils per HPF for 1 subject for all conditions. The mean number of neutrophils per HPF for fMLP for this subject was 60.

Fig. 7. Neutrophil priming for fMLP-stimulated superoxide anion production ($n = 8$ per condition). Priming was elicited by basal medium (BM), LPS (100 mM), and conditioned media obtained from control myotube cultures (CT) and from myotubes exposed to 5, 10, 20, or 30% strain or to a scrape injury protocol (SCP). *Significantly different from CT.

20, and 30% strain failed to increase $\text{O}_2$ from neutrophils adherent to fibronectin relative to control medium (Fig. 9).

Fig. 8. Superoxide anion production from neutrophils in suspension ($n = 11$ per condition). Neutrophils were suspended in fMLP ($2.5 \times 10^{-8}$M) in basal medium (fMLP), basal medium (BM), or conditioned media obtained from control cultures (CT) and from myotubes exposed to 30% strain (30% strain) or a scrape injury protocol (SCP). *Significantly different from CT.
Concentrations that were observed in conditioned media and a concentration that has been reported to elicit neutrophil chemotaxis (5, 17, 23, 24, 42). Positive control concentrations of both rhIL-8 (100 ng/ml) and hTGF-β1 (10 pg/ml) elicited chemotaxis indexes (62.4 ± 9.4 and 27.6 ± 10.0, respectively) that were significantly higher than an index of zero. Concentrations of rhIL-8 at concentrations of IL-8 observed in conditioned media (20, 30, 35, and 70 pg/ml), however, failed to significantly increase the chemotaxis index (2.6 ± 4.4, 4.9 ± 4.9, 1.5 ± 3.4, and 1.5 ± 4.0, respectively; n = 4 per concentration). Similarly, concentrations of hTGF-β1 at active and total TGF-β1 concentrations observed in conditioned media (25, 35, 100, 130, and 170 pg/ml) did not significantly elevate the chemotaxis index (22.5 ± 9.9, 11.0 ± 5.3, 12.6 ± 7.4, 12.5 ± 4.5, and 20.0 ± 9.6, respectively; n = 8 per concentration). A lack of a significant elevation in neutrophil chemotaxis was also apparent when statistically comparing the mean number of neutrophils per high-powered field for rhIL-8 and hTGF-β1 at observed concentrations of IL-8 and TGF-β1 relative to the mean number of neutrophils per high-powered field for the basal medium condition (data not reported).

DISCUSSION

The present study advances our understanding of the immunobiology of skeletal muscle by establishing a novel cell culture model for determining the contribution of skeletal muscle cells to neutrophil responses after mechanical loading and traumatic injury. Both mechanical loading of and injury to myotubes resulted in the release of one or more factors that caused neutrophil chemotaxis and primed neutrophils for ROS production. Neutrophil chemotaxis and priming induced by mechanical loading, however, was dependent on a threshold of strain and occurred in the absence of muscle injury. Neutrophil chemotaxis, but not neutrophil priming, progressively increased with incremental increases in the magnitude of strain. The release of one or more factors from myotubes that increase O2·− production from neutrophils occurred after traumatic injury but not after injurious or noninjurious strain. Together, our results demonstrate that myotubes release one or more factors after mechanical loading and/or injury that differentially influence neutrophil chemotaxis and the stages of neutrophil-derived ROS production. The results from the present study demonstrate for the first time that skeletal muscle cells are an...
important source of neutrophil chemoattractants after mechanical loading and injury.

Neutrophil chemotaxis was sensitive to the magnitude of strain and occurred in the absence of muscle injury. This finding is in agreement with our previous in vivo observations. Specifically, we reported (30, 38) that passive stretching, isometric contractions, and concentric contractions, which do not cause overt injury, elevated neutrophils in skeletal muscle in the hours to days after the activity. The blunted neutrophil chemotaxis elicited by noninjurious relative to injurious strain in the present study is also consistent with the lower concentration of neutrophils after passive stretching and isometric contractions relative to injurious lengthening contractions (38). Thus both mechanical loading and loading-induced injury are potent stimuli for the release of one or more factors from skeletal muscle cells that cause neutrophil chemotaxis.

While migrating to sites of inflammation, human neutrophils can be primed in vivo for ROS production in vitro after injurious and noninjurious exercise (37, 40, 46). In the present study, neutrophil priming for O$_2^*$ production occurred after noninjurious strains (10 and 20%) and was not elevated further by a higher, injurious, strain magnitude (30%). These observations suggest that beyond a threshold of mechanical strain, myotube-induced neutrophil priming is not sensitive to the magnitude of strain or the presence of muscle injury. The lack of specificity and sensitivity for muscle injury may indicate that neutrophil priming merely serves as a preparative stage for ROS production while neutrophils are in transit to their final destination within skeletal muscle. At their final destination, neutrophils may, in the case of overtly injured muscle, encounter soluble agonists and/or immobilized ligands that increase their production of ROS. Interestingly, priming for neutrophil-derived ROS production has been reported to be a reversible event (25). The reversal of priming, in the absence of factors that increase ROS production from neutrophils, may serve as a protective mechanism to prevent neutrophils from injuring cells via the release of ROS (11, 25).

We hypothesized that the microenvironment of skeletal muscle injured by strain and trauma contains one or more soluble factors that increase O$_2^*$ production from neutrophils. Our results partially support this hypothesis by demonstrating that scrape injury, but not injurious nor noninjurious strain, caused myotubes to release factors that increased O$_2^*$ production from neutrophils. The inability of conditioned medium from strain-injured myotubes to increase O$_2^*$ production from neutrophils was not attributable to a suboptimal concentration of potential agonists or to neutrophil priming, because conditioned media failed to increase O$_2^*$ production from unprimed and primed neutrophils. Although previous investigators reported that the potency of TNF-α and GM-CSF is enhanced when neutrophils are adherent to fibronectin (12, 34–36), we also failed to observe a significant influence of strain media on O$_2^*$ production from neutrophils adherent to fibronectin. Together, the ability of injured myotubes to release one or more factors that increase O$_2^*$ production from neutrophils appears to be dependent on the nature of the injury (strain vs. trauma) and/or the magnitude of the injury.

Functional activities of neutrophils are controlled by cytokines, ELR$^+$ CXC chemokines, complement proteins, lipid derivatives, extracellular matrix proteins, and adhesion molecules (8, 15). Interestingly, cultured skeletal muscle has been reported to produce several neutrophil chemoattractants and/or modulators of neutrophil-derived ROS production from each of these categories. For example, protein levels of IL-1, IL-6, IL-8, IL-15, GM-CSF, TGF-β, monocyte chemoattractant protein-1, and fibroblast growth factor have all been reported to be released from cultured skeletal muscle (myoblasts and/or myotubes) under control conditions, after treatment with inflammatory cytokines (e.g., IL-1β and TNF-α) (9, 13, 33, 45), or after mechanical strain (10). Furthermore, cultured myoblasts and/or myotubes express various components of the complement pathway (27) and produce lipid derivatives (e.g., prostaglandins) (52) with chemotactic and ROS-inducing potential. Thus there is compelling evidence that skeletal muscle cells produce an array of factors that are capable of influencing neutrophil responses after mechanical loading and/or injury.

Our initial approach to identifying candidates for the observed responses was to quantify selected factors in conditioned media. To our surprise, IL-8, a potent chemoattractant and primer for ROS production from neutrophils (8), was reduced by mechanical loading and traumatic injury. In addition, rhIL-8 at concentrations of IL-8 observed in conditioned media failed to elicit neutrophil chemotaxis. These later observations are consistent with previous investigators who demonstrated that rhIL-8 causes neutrophil chemotaxis at much higher concentrations (~10 ng/ml–80 μg/ml) than concentrations of IL-8 observed in our conditioned media (10–70 pg/ml) (23, 24). Because TGF-β1, a potent neutrophil chemoattractant and a modest activator of ROS production from adherent neutrophils (3, 42), has been reported to inhibit the synthesis of IL-8 (44), we then measured TGF-β1. The concentration of total TGF-β1 in conditioned media was also reduced by mechanical loading, whereas the active form remained unchanged. Furthermore, hTGF-β1 at concentrations of TGF-β1 observed in conditioned media failed to significantly increase neutrophil chemotaxis. These observations are in agreement with previous investigators who demonstrated that neutrophil chemotaxis in vitro reaches maximal levels at ~1 pg/ml TGF-β1 and declines rapidly with higher concentrations (5, 17, 42). The concentration of TNF-α, which can increase ROS production from adherent, but not suspended, neutrophils (12, 35, 43), was not influenced by mechanical loading or injury. The concentrations of TNF-α observed in conditioned media (<20 pg/ml) were much lower than concentrations (0.1–100 ng/ml) that have been reported toincrease ROS production from neutrophils (12, 35, 43). Together, our observations suggest that factors other than IL-8, TGF-β1, and TNF-α are probably orchestrating the observed neutrophil responses after mechanical strain and/or injury to myotubes.

The present study demonstrates that mechanical loading and/or injury influences the immunobiology of skeletal muscle cells in a manner that differentially influences neutrophil chemotaxis and the stages of neutrophil-derived ROS production. This sophisticated level of interplay may serve as a mechanism by which the microenvironment of skeletal muscle dictates the concentration and the functional outcome of neutrophils in injured and noninjured skeletal muscle after exercise. Further work is needed to identify the skeletal muscle-derived factors that orchestrate neutrophil chemotaxis, priming, and ROS production after mechanical loading and traumatic injury. Once the skeletal muscle-derived factors have been identified, therapeutic and/or pharmacological strategies could be devel-
opied to manipulate the immunobiology of skeletal muscle to minimize any negative consequences of neutrophils in skeletal muscle.

GRANTS

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